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Factores climáticos y agronómicos que determinan la incidencia y distribución geográfica de nematodos fitoparásitos en olivar en Andalucía

CLIMATIC AND AGRONOMIC FACTORS DETERMINING THE INCIDENCE AND GEOGRAPHIC DISTRIBUTION OF PLANT-PARASITIC NEMATODES IN OLIVE IN ANDALUSIA

TESIS DOCTORAL

Don Antonio J. Archidona Yuste

Para la obtención del

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Directores:

Dr. Juan A. Navas-Cortés

Dr. Pablo Castillo

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TITULO: *FACTORES CLIMÁTICOS Y AGRONÓMICOS QUE DETERMINAN LA INCIDENCIA Y DISTRIBUCIÓN GEOGRÁFICA DE NEMATODOS FITOPARÁSITOS EN OLIVAR EN ANDALUCÍA*

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TÍTULO DE LA TESIS:
Factores climáticos y agronómicos que determinan la incidencia y distribución geográfica de nematodos fitoparásitos en olivar en Andalucía

DOCTORANDO/A: Antonio Archidona Yuste

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Durante el período pre-doctoral el doctorando, ha cubierto los objetivos previstos en el calendario programado para este trabajo de investigación; y el grado de cumplimiento de los diferentes objetivos y tareas se ajusta al previsto en su proyecto de Tesis Doctoral.

Las investigaciones realizadas incluyen diversos aspectos sobre incidencia y distribución de nematodos fitoparásitos en olivar en Andalucía. Para ello el Doctorando ha adquirido formación sólida en técnicas de muestreo, identificación morfométrica y molecular de nematodos fitoparásitos, así como técnicas de análisis estadísticos avanzados. Los resultados obtenidos hasta el momento han permitido la elaboración y publicación de CUATRO artículos indexados ISI-JCR y UNO actualmente en revisión, todas ellas catalogadas dentro del primer cuartil (Q1), así como dos comunicaciones orales y posters presentados en el Congreso de la Sociedad Española de Fitopatología y The Mediterranean Phytopathological Union. Adicionalmente, el doctorando ha colaborado en investigaciones del Grupo de investigación que han conllevado a la autoría o co-autoría de 34 artículos indexados ISI-JCR y un artículo en una revista de divulgación del sector agrícola. Entre los resultados más sobresalientes del proyecto de Tesis Doctoral del doctorando, cabe destacar la identificación y descripción de un total de 14 nuevas especies para la ciencia de nematodos fitoparásitos aisladas de la rizosfera del cultivo de olivo en Andalucía. Además, se han determinado asociaciones entre variables ambientales y patrones de comunidades de nematodos fitoparásitos.

La labor realizada por el doctorando durante el período pre-doctoral le ha permitido asentarse con firmeza en el Programa de investigación que constituye su Tesis Doctoral. Durante este tiempo, el doctorando ha llevado a cabo de forma eficiente y entusiasta las tareas asignadas a su proyecto de Tesis Doctoral, y ha colaborado de forma muy activa en las diversas investigaciones que se estaban llevando a cabo en el Grupo de investigación durante esta etapa. Asimismo, el doctorando ha asistido con aprovechamiento a seminarios y conferencias de carácter científico, y a cinco cursos avanzados de formación que le han sido de gran utilidad en su proyecto de Tesis Doctoral.

Artículos Publicados y en revisión:

1. **ARCHIDONA-YUSTE, A.**, NAVAS-CORTÉS, J.A., CANTALAPIEDRA-NAVARRETE, C., PALOMARES-RIUS, J.E., & CASTILLO, P. 2016. Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematoda:

Longidoridae) as inferred from morphometrics and molecular markers. *ZOOLOGICAL JOURNAL OF THE LINNEAN SOCIETY* 176, 231-265

2. **ARCHIDONA-YUSTE, A.**, NAVAS-CORTÉS, J.A., CANTALAPIEDRA-NAVARRETE, C., PALOMARES-RIUS, J.E., & CASTILLO, P. 2016. Unravelling the biodiversity and molecular phylogeny of needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) in olive and a description of six new species. *PLoS ONE* 11e0147689, 1-53.

3. **ARCHIDONA-YUSTE, A.**, NAVAS-CORTÉS, J.A., CANTALAPIEDRA-NAVARRETE, C., PALOMARES-RIUS, J.E., & Castillo, P. 2016. Molecular phylogenetic analysis and comparative morphology resolve two new species of olive-tree soil related dagger nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) from Spain. *Invertebrate Systematics* 30, 547-565.

4. **ARCHIDONA-YUSTE, A.**, NAVAS-CORTÉS, J.A., CANTALAPIEDRA-NAVARRETE, C., PALOMARES-RIUS, J.E., & CASTILLO, P. 2016. Remarkable diversity and prevalence of dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches. *PLoS ONE* 11e0165412, 1-54.

5. **ARCHIDONA-YUSTE, A.**, WIEGAND, T., CASTILLO, P. & NAVAS-CORTÉS, J.A. 2018. Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olives in Southern Spain. *AGRICULTURE ECOSYSTEMS AND ENVIRONMENT* under review (minor revision).

6. **ARCHIDONA-YUSTE, A.**, WIEGAND, T., CASTILLO, P. & NAVAS-CORTÉS, J.A. 2018. Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in Southern Spain. *DATA IN BRIEF* under review.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 2 de noviembre de 2018

Firma del/de los director/es

Fdo.: Dr. Juan A. Navas Cortés

Fdo.: Dr. Pablo Castillo Castillo

A mis padres, Antonio y Francisca,
mi hermana Verónica,
y a Julia

AGRACEDIMIENTOS

Tengo que admitir que estos párrafos empecé a redactarlos una vez finalizado el documento que empieza en las siguientes páginas. No por ello estas palabras las considero de menor importancia sino lo contrario, ya que esta tesis es el fruto de un intenso trabajo e ilusión no sólo de una persona, sino de muchas que directamente e indirectamente, y en mayor o menor grado han influido para que la realización de esta tesis haya sido posible. A tod@s quiero agradecerles su esfuerzo con simples palabras, pero sinceras y llenas de aprecio.

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¡¡Muchas gracias!!

ABSTRACT

Unravelling of soil nematode biodiversity is an essential task in order to increase the knowledge about ecological phenomenon from the evolutionary, biogeographical and physical processes in soil ecosystems. In a phytopathological context, deciphering the diversity of plant-parasitic nematodes (PPN) infesting soils from an agricultural ecosystem is an essential task in order to design useful management practices for controlling potential nematode diseases. Overall, most of PPN have a broad range of host plants, natural plants or crops including annual, biannual or perennial plants. Similarity to their wide host range as generalist nematodes, there are plants that can host a wide range of PPN, as is the case for olive trees. In fact, olives, both in wild and cultivated forms, serve as hosts to a wide diversity of PPN, including endoparasitic, semiendoparasitc and ectoparasitic species. Approximately 50% of the total surface area of Andalusia region is covered with natural and forest vegetation, and 44% by agricultural areas which are associated with olive orchards, cereal crops and vineyards. Although cultivated olive is extensively grown in the Mediterranean Basin, in Andalusia this cultivated non-tropical fruit tree covers more than 1.6 million ha accounting for 19% of the total region surface area in an impressive monoculture, being culturally and economically very relevant in this region. In this research, we aimed to unravel the diversity of PPN associated with cultivated olive in southern Spain through the largest nematode sampling effort on olive. We conducted a systematic survey comprising 376 commercial olive orchards covering the diversity of cropping systems that characterize the entire olive area of Andalusia, including agroforestry stands, traditional groves and new intensive orchards as well as a wide range of ecological gradients related with topography, soil and climate.

A total of 128 PPN species, belonging to 38 genera and 13 families, were recorded by using integrative taxonomy based on morphological and molecular approaches, which highlights a high taxonomical diversity of PPN communities and resulting in the greatest taxonomical diversity detected in cultivated olive. In addition, this increases the number of PPN associated with olive, being estimated about 250 species worldwide. Overall, the three

most prevalent families in cultivated olive from Andalusia were Tylenchidae, Paratylenchidae and Criconematidae, and the nematode families with the highest average nematode densities were Meloidogynidae, Hoplolaimidae and Paratylenchidae. And the family with the highest number of species was Longidoridae with a total of 28 species identified. On the other hand, the PPN abundance in olive ranged from 7 to about 20,000 nematode specimens per 500 cm^3 of soil. *Helicotylenchus oleae* and *Ogma rhombosquamatum* showed the highest nematode abundance with 19,796? and 9,800 nematodes per 500 cm^3 of soil, respectively. The nematode species most prevalent were *Merlinius brevidens* (72.6%) and *Xiphinema pachtaicum* (70.4%).

This research also makes available the first detailed analysis of the diversity and distribution of PPN belonging to Longidoridae infesting wild and cultivated olive in a wide-region in southern Spain as Andalusia, providing new insights of this family associated with olive in Mediterranean conditions. Although the most important nematodes economically include endoparasitic species such as root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.), the phytopathological importance of the ectoparasitic nematodes belonging to the family Longidoridae not only lies in its wide range of host and cosmopolitan distribution, but some species of this genus are vectors of several important plant viruses (genus *Nepovirus*, family Comoviridae), causing significant damage to a wide range of crops. The family Longidoridae is considered as one of the most evolved and diverse nematode group within the phylum Nematoda. Longidorid nematodes, including the genera *Xiphinema* and *Longidorus*, are also characterized by having a high intra- and inter-specific morphological homogeneity and pronounced phenotypic plasticity, and the existence of complex cryptic species. Species discrimination in this group has classically been based mainly on morphology and morphometrics of diagnostic features. However, morphologically based species characterization is complicated by a high degree of intraspecific variability within morphometrics, as well as small interspecific differences that lead to substantial overlapping among species and increase the risk of species miss-identification. Thus, we demonstrate the importance of using integrative taxonomic identification highlighting the time-consuming aspect and difficulty of correct identification at species

level within the family Longidoridae. In addition, we provide a new insight in the identification of *Xiphinema americanum*-group species including statistical multivariate methods to the custom integrative taxonomical approach.

We reveal a remarkable diversity and distribution of longidorid species infesting soils of olive trees (cultivated and wild olive) in Andalusia with a total identification of 32 and 13 species for *Xiphinema* and *Longidorus*, respectively; and diversity indexes were significantly affected by olive type. The species most prevalent and abundant within this family was *Xiphinema pachtaicum*, nematode species belonging to the *X. americanum*-group. As a consequence of this, the overall nematode abundance recorded in each sampling point was significantly higher for the *X. americanum*-group than *X.* non-*americanum*-group. However, the exceptional diversity recorded for the genus *Xiphinema* was mainly associated with the *X.* non-*americanum*-group that showed a wide distribution in the whole surface occupied by olive in Andalusia, especially in the wild olive areas. In addition, the overall nematode abundance recorded in each sampling was also significantly higher for cultivated than wild olive. The results obtained in relation to the diversity detected between wild and cultivated olive revealed new insights about the influence of the natural environment and cultivated in the diversity and distribution of species belonging to family Longidoridae. Although differences in this regard were observed in the genus *Longidorus*, the analysis of biodiversity in the genus *Xiphinema* through the application of gamma diversity indexes (e.g. species richness index, Shannon or Hill indexes, among others) showed a significant influence by olive forms (cultivated and wild olive). In fact, our findings revealed a lower value of diversity indexes for wild than cultivated olive, especially in species index.

This research also provides a complete characterization of 15 new species belonging to the family Longidoridae, providing also molecular markers for already known longidorid species for precise and unequivocal diagnosis in order to differentiate virus vector or quarantine species. The new species described in this Doctoral Thesis included 6 for the genus *Longidorus* (*Longidorus indalus* n. sp., *Longidorus macrodorus* n. sp., *Longidorus onubensis* n. sp., *Longidorus silvestris* n. sp., *Longidorus vallensis* n. sp., and *Longidorus wicuolea* n. sp.), and 9 for the genus

ABSTRACT

Xiphinema (*Xiphinema andalusiense* n. sp., *Xiphinema astaregiense* n. sp., *Xiphinema celtiense* n. sp., *Xiphinema iznajarense* n. sp., *Xiphinema macrodora* n. sp., *Xiphinema mengibarense* n. sp., *Xiphinema oleae* n. sp., *Xiphinema plesiopachtaicum* n. sp., and *Xiphinema vallense* n. sp.). In addition, this study reveal a remarkable diversity of nematodes belonging to the family Longidoridae in the context of number of species recorded, but also, the diversity detected here is relevant in a wide variability of the morphological characters within the species identified. In fact, data obtained in this Doctoral Thesis expands the body size and odontostyle length ranges known for *Xiphinema* spp. with the new description of *Xiphinema macrodora* n. sp., the *Xiphinema* sp. with the largest odontostyle and body length. Additionally, *X. macrodora* n. sp. co-occurred with another large longidorid nematode species with a long stylet (*L. macrodorus* n. sp.) in the same sample point. On the other hand, the description of the new species *Xiphinema oleae* n. sp. is considered as a phenomenon not common within the genus *Xiphinema*, since the morphology that underlies this species belonging to the *X.* non-*americanum*-group with the presence of a true "Z organ" determining the appearance of the uterine differentiation and including this nematode within the morphospecies "Group 4", results in a rare event since the low number of species with such characteristics. In summary, these results strengthen the hypothesis that describes the south of the Iberian Peninsula as a possible speciation area of the family Longidoridae, given not only by the extraordinary diversity in terms of number of species but also the morphological variability detected.

Although considerable work has been performed on nematode ecology, relatively little attention has been paid to investigate the ecological factors controlling the spatial diversity of PPN communities. Although several studies have found environmental and agronomic factors driving the diversity of PPN in commercial olive orchards, the information is incomplete, especially in the case of the olive growing area of the south of Iberian Peninsula (e.g. Andalusia). In fact, key questions such as what is the influence of stochasticity (namely unexplained variation or neutral processes); or what is the influence of spatial structure among others have not been addressed. Furthermore, most of the ecological studies based on the spatial distribution of PPNs have been developed using alpha or gamma diversity as descriptive variables of biological biodiversity.

However, compared with beta diversity these approaches do not allow testing hypotheses about the processes underlying species distribution and biodiversity since disregard species identity. Therefore, we investigated how different sets of variables describing the environment (i.e. soil, above-ground environment, and agricultural management) and spatial structure influenced controlled the variation of community composition (e.g. beta diversity) and species richness of PPNs infesting the rhizospheric soil from the 376 commercial olive orchards mentioned above. Overall, a total of 52 explanatory variables were considered and related with climate, topography, soil and agronomic management. Spatial structure was included into ecological models by characterizing spatial relationships of sampling design as spatial descriptors or covariates, using the principal coordinate of neighbour matrices (PCNM) method. Beta diversity was computed as the total variance of the transformed abundance-biomass community data to PPN species level, which allowed to partition the overall diversity into the contribution of single sites (LCBD; ecological uniqueness of sites in terms of community composition) and into the contribution of individual species (SCBD: the relative importance of each species in affecting beta diversity). In addition, LCBD index was tested by influence of environmental and/or general characteristics of the PPN community, since this index could indicate degraded sites or sites with particular ecological conditions. SCBD index was also tested with general nematode species traits, including prevalence, density range or nematode biomass. To assess the relative and shared contributions of the different environmental factors and spatial structure the variation in PPN species richness and community composition, we used variation partitioning approach, which also allowed to determine the influence relative to deterministic and stochasticity component. The methodology applied in this research is considered a novelty in the scientific studies of PPN communities.

In brief, our findings were surprising in contrast to widely accepted view in this sense described in the literature. In fact, contrary to the expectations that soil and management would largely determine PPN community structure, we found that more than two-thirds of the variation remained unexplained. Then, we found a prominent role of stochasticity in structuring PPN communities with 85% of the variation unexplained for beta diversity and 67% for species richness. Space and soil variables were the most

important effect on both species richness and beta diversity. However, effects of agronomic practices on species richness were lower than expected, whereas they had no effect on beta diversity. Pure spatial component (i.e. that comprises spatial component independent of any measurable environmental variables) was the most influent variable set for beta diversity, and soil component for species richness. Overall, the variance explained by the pure contribution of environmental variables (including climatic and topographic variables) was negligible, relatively small compared to that explained by the other sets of variables. The most influent variables explanatory variables were soil chemical properties including CEC, pH, extractable P, showing this a strong effect on the variation of species richness. In addition, we found relatively high levels of shared contributions of the different sets of variables, especially with space, indicating spatial gradients in the environmental variables. In fact, the influence of agronomic management was spatially structured and/or correlated with other explanatory variables but no effect by the pure management component. However, we found an effect of irrigation regimen (i.e. irrigated) and below canopy tree management (i.e. presence of natural vegetation below tree canopy) in structuring the variation of species richness. Finally, this research revealed substantial differences between the effects of overall environmental variables on beta diversity and species richness. First, the variation explained on species richness was higher than beta diversity, which is against the general pattern detected in above-ground systems. And second, while the pure management component did not influence beta diversity, the pure component of management practices influenced variation of species richness.

Species contributions to beta diversity (SCBD) were positively correlated with nematode prevalence and density range, suggesting that SCBD could be related with niche position as reported in other ecosystems. Therefore, dominant (i.e. most prevalent) PPN species showing the largest abundance variation among sites, such as the genera *Helicotylenchus* and *Xiphinema*, may be a suitable indicator of fluctuations on specific environmental properties in agricultural ecosystems. Local contributions to beta diversity (LCBD) was positively correlated with the total nematode biomass in each site in contrast to the species richness and nematode abundance as expected. Thus, LCBD may result in distinctly perceptible

changes in PPNs assemblages based on the close relationship between soil organism size and ecological gradients. Finally, commercial olive orchards with significant values for LCBD (i.e. highest values) were grouped into two clearly separated areas, suggesting strong habitat filtering mechanisms. In fact, we found significant relationships among LCBD values and environmental and agronomic practices.

In summary, this research reveals novel insights in the spatial structuring of PPN diversity and show that their beta diversity is less structured by space and environmental factors as compared to other organism types. And novel insights are revealed about the pure effect of environment and agronomic practices on diversity and distribution of PPN infesting soils from agricultural ecosystems. However, an interesting open question left for future studies could be if PPNs of wild forms of olives would show more biological structure in beta diversity than that found here for the cultivated forms. These results would allow increasing the reliability of the management practices of these parasites in agricultural ecosystems.

RESUMEN

El análisis de la biodiversidad de la nematofauna presente en el suelo es esencial para incrementar el conocimiento sobre los procesos ecológicos que subyacen de la evolución y biogeografía de los organismos que viven en él. En un contexto fitopatológico, descifrar la diversidad de nematodos fitoparásitos (NF) que infestan los suelos de un ecosistema agrícola es una tarea esencial para el diseño eficaz de prácticas de manejo para el control de potenciales procesos de enfermedad que puedan ser ocasionados por nematodos. En general, la mayoría de las especies de NF que podemos encontrar en un agroecosistema son capaces de parasitar una amplia gama de plantas huésped incluyendo plantas silvestres y cultivadas (anuales o perennes). Asimismo, existen plantas que pueden albergar una amplia gama de especies de NF, como es el caso de la planta de olivo. De hecho, el olivo, ya sea en la forma silvestre o cultivada, ha sido descrito como un excelente huésped de una multitud de especies de NF, incluyendo especies endoparásitas, semiendoparásitas y ectoparásitas. Aproximadamente el 50% de la superficie total de la comunidad autónoma de Andalucía está ocupada por ecosistemas naturales y/o forestales, y el 44% por ecosistemas agrícolas, entre los cuales predominan los cultivos del olivo, vid y cereal. Aunque el olivo es el cultivo por excelencia de la Cuenca Mediterránea, la superficie ocupada por este cultivo en Andalucía asciende hasta 1,6 millones de hectáreas, lo que supone el 19% de la superficie total de la comunidad autónoma. Este hecho hace que el cultivo del olivo presente una elevada importancia económica y social en esta región. Por todo ello, el objetivo finalista planteado en la presente Tesis Doctoral fue determinar la diversidad de NF asociados al olivo que infestan el suelo en Andalucía, a partir de un muestreo sistematizado que fue caracterizado como el mayor esfuerzo nematológico descrito hasta la fecha para este cultivo. Para ello, se realizaron prospecciones fitonematológicas en un total de 376 parcelas comerciales de olivo cultivado, lo que permitió abarcar la amplia variabilidad ambiental en relación a propiedades climáticas, edáficas y topográficas, así como todos los sistemas de producción agrícola que exhibe el olivar andaluz.

RESUMEN

Se identificaron un total de 128 especies de NF, incluidas en 38 géneros y 13 familias, mediante un diagnóstico integrativo basado en estudios morfológicos y técnicas moleculares, lo que resultó en la mayor diversidad taxonómica de NF detectada en el olivo cultivado descrita hasta el momento. Además, este estudio ha incrementado la diversidad de NF asociados al olivo, incrementándose esta diversidad hasta las 250 especies a nivel mundial. Las tres familias detectadas con una mayor prevalencia fueron Tylenchidae, Paratylenchidae y Criconematidae; y aquellas con el mayor promedio de densidad encontrada fueron Meloidogynidae, Hoplolaimidae y Paratylenchidae. Otro dato de interés indicó que la familia Longidoridae presentó el mayor número de especies identificadas con un total de 28 especies diferentes. Por otro lado, la abundancia detectada en las parcelas comerciales de olivo varió entre 7 hasta cerca de los 20.000 especímenes por 500 cm^3 de suelo. *Helicotylenchus oleae* y *Ogma rhombosquamatum* fueron las especies que mostraron la mayor densidad con 19.796 y 9.800 nematodos por 500 cm^3 de suelo, respectivamente. Las especies con una mayor prevalencia fueron *Merlinius brevidens* (72.6%) y *Xiphinema pachtaicum* (70.4%).

La presente Tesis Doctoral también proporciona nuevos conocimientos sobre la familia Longidoridae en la cuenca Mediterránea mediante el primer estudio detallado sobre la diversidad y distribución de especies de esta familia infestando suelos de olivo cultivado y silvestre en la comunidad autónoma de Andalucía. Aunque las especies de NF con un mayor impacto económico son las especies endoparásitas pertenecientes a los géneros *Meloidogyne*, *Heterodera* y *Globodera*, la importancia fitopatológica de los NF de la familia Longidoridae no solo subyace de su amplia gama de huéspedes y su distribución, sino porque algunas especies son vectores de virus fitopatógenos (género *Nepovirus,* familia Comoviridae), causando daños significativos y pérdidas de producción en multitud de cultivos. La familia Longidoridae es considerada como uno de los grupos de NF más evolucionados y diverso del filo Nematoda. Los nematodos longidóridos, incluidos en los géneros *Xiphinema* y *Longidorus*, son caracterizados también por presentar una elevada homogeneidad morfológica y una remarcable plasticidad fenotípica entre especies diferentes, así como la existencia de un elevado número de complejos de especies crípticas. La delimitación de especies dentro de la familia Longidoridae se ha basado en

el uso de caracteres morfológicos y morfométricos. Sin embargo, la caracterización morfológica mediante el uso de medidas de los caracteres de diagnóstico es sumamente complicada aun siendo efectuada por parte de personal cualificado. Esto se debe al efecto del solapamiento morfológico entre especies debido al alto grado de variabilidad intraespecífica y las pequeñas diferencias entre especies en las medidas de los caracteres diagnósticos. De este modo, resulta fácil cometer errores de identificación entre especies dentro de la familia Longidoridae. En este estudio demostramos la utilidad del uso de la taxonomía integrativa disminuyendo el tiempo requerido para realizar un diagnóstico adecuado a nivel de especie, a pesar de la notable dificultad que caracteriza este aspecto en la familia Longidoridae. Además, este trabajo proporciona una nueva perspectiva en la identificación de especies del grupo *americanum* del género *Xiphinema* incluyendo métodos estadísticos de análisis multivariante en un enfoque de diagnóstico integrativo.

La diversidad y distribución de especies de nematodos longidóridos infestando el suelo del olivo (tanto en su forma cultivada como silvestre) en Andalucía fue extraordinaria. Se identificaron un total de 32 y 13 especies diferentes para el género *Xiphinema* y *Longidorus*, respectivamente; y los índices de diversidad estuvieron influidos por el tipo de olivo. De todas las especies de nematodos longidóridos identificadas, la que presentó la mayor prevalencia y fue detectada con la máxima densidad fue *Xiphinema pachtaicum.* Como consecuencia, la abundancia total de especies de longidóridos registrada en cada punto de muestreo fue significativamente mayor para el grupo *americanum* que para el grupo no *americanum* del género *Xiphinema*. Sin embargo, la excepcional diversidad detectada para el género *Xiphinema* en cuanto a número de especies diferentes fue asociada principalmente al grupo no *americanum* que a su vez fue distribuido ampliamente por toda la superficie de Andalucía, especialmente para aquellas zonas donde el olivo silvestre está presente. Es necesario indicar que la abundancia total registrada en cada punto de muestro para la familia Longidoridae fue significativamente superior en el olivo silvestre que en el cultivado. En este sentido, los resultados obtenidos en cuanto a las diferencias en la diversidad y abundancia de nematodos longidóridos detectada entre el olivar silvestre y cultivado revelaron nuevos conocimientos sobre la influencia de la agricultura y el ambiente natural en

la diversidad y distribución de nematodos longidóridos. Aunque se observaron diferencias en este sentido en el caso del género *Longidorus*, el análisis de la biodiversidad en el género *Xiphinema* a partir del uso de índices de diversidad gamma (p.ej. el índice de diversidad Shannon, Hill´s, entre otros) reveló una influencia significativa por parte del tipo del olivo. De hecho, los datos analizados en el presente estudio revelaron valores menores en los índices de diversidad utilizados en el caso del olivar silvestre, especialmente para el índice de riqueza de especies.

La presente Tesis Doctoral también proporciona una completa caracterización morfológica y molecular de un total de 15 nuevas especies pertenecientes a la familia Longidoridae, además de facilitar por primera vez marcadores moleculares para un amplio conjunto de especies de nematodos longidóridos ya descritas previamente. Las nuevas descripciones de especies incluyeron 6 para el género *Longidorus* (*Longidorus indalus* n. sp., *Longidorus macrodorus* n. sp., *Longidorus onubensis* n. sp., *Longidorus silvestris* n. sp., *Longidorus vallensis* n. sp., y *Longidorus wicuolea* n. sp.), y 9 en el caso del género *Xiphinema* (*Xiphinema andalusiense* n. sp., *Xiphinema astaregiense* n. sp., *Xiphinema celtiense* n. sp., *Xiphinema iznajarense* n. sp., *Xiphinema macrodora* n. sp., *Xiphinema mengibarense* n. sp., *Xiphinema oleae* n. sp., *Xiphinema plesiopachtaicum* n. sp., y *Xiphinema vallense* n. sp.). Además, este estudio revela una extraordinaria diversidad en cuanto a número de especies diferentes detectadas en la familia Longidoridae, pero también es necesario indicar que se detectó una relevante variabilidad morfológica entre las especies identificadas. De hecho, los resultados obtenidos en esta Tesis Doctoral incrementan el rango conocido del tamaño del cuerpo y longitud del odontostilo en el caso del género *Xiphinema* con la nueva descripción de la especie *Xiphinema macrodora* n. sp. caracterizada por presentar el odontostilo y el cuerpo más largo descrito hasta la fecha para este género. Esta especie fue encontrada en el mismo punto de muestro donde fue detectada la especie con el odontostilo más largo dentro de la familia Longidoridae, es decir *L. macrodorus* n. sp. Por otro lado, la descripción de la nueva especie *Xiphinema oleae* n. sp. es considerada como un hecho no común en el género ya que la presencia de un órgano Z verdadero, como elemento que determina y caracteriza la diferenciación uterina que exhibe esta especie incluida en el grupo de morfoespecies 4,

resulta en un hecho poco frecuente en este género. En resumen, los resultados obtenidos aquí están de acuerdo con la hipótesis que describe a la Península Ibérica como una posible área de especiación de la familia Longidoridae, no sólo dado por la elevada presencia de especies diferentes sino por la notable variabilidad morfológica detectada entre ellas.

Aunque se ha realizado un considerable esfuerzo en la ecología de los nematodos edáficos, muy pocos estudios se han enfocado para descifrar los factores ambientales que determinan la distribución espacial de comunidades de NF en ecosistemas agrícolas. Aunque en la bibliografía podemos encontrar varios trabajos científicos que identifican potenciales factores ambientales y agronómicos que estructuran la diversidad de NF en parcelas comerciales de olivo cultivado, la información al respecto es incompleta, especialmente en el caso de la zona olivarera de Andalucía. De hecho, una serie de cuestiones clave en un contexto ecológico permanecen sin ser respondidas en la bibliografía en este sentido. Estas preguntas pueden ser las siguientes: ¿cuál es la influencia de la estocasticidad (es decir, variación espacial no explicada o procesos relacionados con la Teoría Neutral Unificada) en la estructura espacial en la diversidad de NF? O ¿cuál es la influencia de la estructura espacial? etc. A todo ello hay que añadir que la mayoría de los estudios ecológicos basados en la distribución espacial de NF han sido aplicados mediante el uso de índices gamma y alfa como variables descriptoras de la diversidad encontrada. Sin embargo, en comparación con el uso de índices de diversidad beta, estos enfoques citados anteriormente no permiten testar hipótesis sobre los procesos que realmente estructuran la distribución y la biodiversidad de especies de NF, ya que no tienen en cuenta la identidad de las especies identificadas en cada punto de muestreo. Por todo ello, otra parte de esta Tesis Doctoral estuvo relacionada con el análisis de la influencia de varios bloques de variables que describieron el ambiente (p.ej. suelo, clima, topografía, y manejo agronómico) y la estructura espacial en la variabilidad de las comunidades de NF que infestan suelos de olivo cultivado en Andalucía mediante el uso de variables espaciales y la aplicación de índices de diversidad beta con el fin de comparar los resultados con aquellos obtenidos con el índice de riqueza de especies como medida de diversidad gamma. Para ello, se tuvieron en cuenta un total de 52 variables, relacionadas con el clima, topografía, suelo, y manejo

agronómico. Por otro lado, la estructura espacial fue incluida mediante la incorporación de la fluctuación especial existente en el diseño del maestro a partir de covariables espaciales, usando el método ampliamente reconocido en los estudios ecológicos como es el “método de coordenadas principales de matrices vecinas (PCNM). La diversidad beta fue calculada como la varianza de la matriz transformada de la comunidad detectada como abundancia-biomasa. Esto permitió dividir la contribución relativa por parte de las especies (índice SCBD) de NF que componen a la comunidad y puntos de muestreo (índice LCBD) sobre la variación total en la composición de las comunidades de especies detectadas. Además, la variación espacial en el índice LCBD fue testada por la influencia de factores ambientales y/o características generales de la comunidad de NF, ya que este índice podría estar relacionado con la presencia de áreas degradadas o puntos de muestro con condiciones ecológicas particulares. Por otro lado, el índice SCBD fue testado con la presencia de características específicas de las especies de nematodos, tales como prevalencia, rango de densidad detectado y la biomasa de cada especie de nematodo. Con el fin de determinar la influencia relativa y compartida entre los bloques de variables en la diversidad beta y variación en el índice de riqueza, utilizamos técnicas de partición de la varianza, lo que permitió calcular la influencia de los procesos deterministas y estocásticos. La metodología aplicada en esta investigación es considerada como una novedad en el ámbito científico relacionado con las comunidades de NF.

Nuestros resultados fueron sorprendentes en contra del punto de vista aceptado en este sentido en la bibliografía. De hecho, en contra de lo esperado, de que el suelo y el manejo agronómico determinaran en gran parte la estructura espacial de las comunidades de NF, encontramos que más de dos tercios de la variación no fueron explicados a partir de la amplia gama de factores potenciales tenidos en cuenta. Es decir, nuestros resultados revelaron un papel importante de la estocasticidad en la variabilidad espacial de las comunidades de NF ya que un 85% de la variación no fue explicada en el caso de la diversidad beta, y un 67% en el caso del índice de riqueza de especies. Las variables del espacio y suelo mostraron el efecto más importante tanto en la diversidad beta como riqueza de especies. Sin embargo, el efecto de las variables que describen el manejo agronómico fue menor del esperado en la riqueza de especie,

mientras que no mostraron ningún efecto sobre la diversidad beta lo cual fue sorprendente en contra de lo esperado y descrito en la bibliografía. La influencia pura del componente espacial (es decir, influencia independiente de cualquier relación y/o correlación con cualquier variable ambiental) fue la mayor que se detectó entre todos los conjuntos de variables tenidos en cuenta en este estudio. En general, la variación explicada por la contribución pura de las variables ambientales que determinan el ambiente sobre el suelo (es decir, variables relacionadas con el clima y topografía) fue insignificante, notablemente inferior en comparación con la variación explicada por el resto de conjuntos de variables usadas en el estudio. Las variables que más influyeron fueron las propiedades químicas tales como el CEC, pH y el P asimilable, mostrando esta última un efecto significativo sobre la variación en el índice de riqueza de especies. Además, encontramos niveles relativamente altos de contribuciones compartidas de los diferentes conjuntos de variables, especialmente con el espacio, que indican gradientes espaciales en las variables ambientales. De hecho, la influencia del manejo agronómico se estructuró espacialmente y/o se correlacionó con otras variables usadas, ya que el componente del manejo agronómico no mostró efecto puro en la diversidad beta. No obstante, nuestros resultados mostraron un efecto significativo del régimen de riego (en este caso la presencia de riego) y manejo bajo la copa de los árboles (en este caso la presencia de vegetación natural) en la estructuración de la variación de la riqueza de especies. Finalmente es necesario indicar que este estudio encontró diferencias sustanciales entre los efectos de las variables ambientales en general (es decir, clima, suelo y manejo agronómico) sobre la diversidad beta y la riqueza de especies. En primer lugar, la variación explicada en la riqueza de especies fue mayor que la detectada para la diversidad beta, lo cual está en contra del patrón general observado en otros sistemas sobre el suelo. Y, en segundo lugar, mientras que el manejo agronómico no mostro un efecto puro en la diversidad beta, dicho bloque de variables si mostro una influencia en la variación de la riqueza de especies.

Como se ha indicado con anterioridad y con objeto de identificar áreas y especies de particular interés, la diversidad beta se dividió en dos índices relacionados con la contribución de los puntos de muestreo (LCBD) y las especies (SCBD) en la variación de comunidades de NF. En este sentido,

el índice SCBD se correlacionó con la prevalencia y rango de densidad de NF, lo que sugiere que el SCBD podría estar relacionado con la posición de nicho ecológico como ocurre en otros sistemas. Por lo tanto, especies prevalentes que a su vez han sido encontradas con una amplia variación en su densidad de población entre los puntos de muestro, como es el caso de especies pertenecientes a los géneros *Helicotylenchus* y *Xiphinema*, podrían ser usadas como indicadores adecuados de fluctuaciones de propiedades ambientales específicas en ecosistemas agrícolas. Por otro lado, el índice LCBD mostró una correlación positiva con la biomasa total de nematodos en cada punto de muestro en contra del índice de riqueza y la abundancia numérica de nematodos como era de esperar según lo citado en la bibliografía en este sentido. De este modo, la variación de este índice podría indicar cambios perceptibles en los ensamblajes en las comunidades de NF dada la estrecha relación entre el tamaño del organismo del suelo con ciertos gradientes ecológicos. Finalmente, las parcelas comerciales de olivo cultivado que mostraron valores significativos para el índice LCBD (es decir, aquellos con los valores más altos) se agruparon en dos áreas claramente separadas, lo que surgiere una fuerte asociación de factores ambientales. De hecho, encontramos relaciones significativas entre la variación del índice LCBD con factores ambientales y ciertas prácticas agrícolas.

En resumen, la investigación llevada a cabo en esta Tesis Doctoral en el sentido del conocimiento real de los factores que determinan la biodiversidad de NF en el agroecosistema del olivar revela nuevos conocimientos sobre la estructura espacial de comunidades de estos organismos, y muestra que su diversidad beta está determinada en menor grado por la estructura espacial y factores ambientales en comparación con otros organismos. Además, se aportan nuevas ideas sobre el efecto puro del ambiente y el manejo agronómico en la diversidad y distribución de NF que infestan suelos de los ecosistemas agrícolas. No obstante, una pregunta queda establecida para futuros estudios y trata sobre la comparación de los factores que determinan las comunidades de NF en el olivar silvestre, considerado como ecosistema natural, en comparación con los resultados obtenidos en el olivar cultivado. Y en definitiva, los resultados mostrados en la presente investigación permitirán aumentar la

fiabilidad en el diseño del manejo y control de estos parásitos de plantas en los agroecosistemas.

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INTRODUCCIÓN GENERAL Y OBJETIVOS

En los últimos años, el olivar está siendo objeto de una notable erosión de su estatus fitosanitario, al cual hay que añadir una reducción de la calidad biológica de los suelos que amenazan seriamente su estabilidad y productividad. Además de la grave situación debida a la Verticilosis y del riesgo que implica la presencia de la bacteria *Xylella fastidiosa* en Europa, hay que destacar varias enfermedades que han incrementado marcadamente su gravedad en las últimas décadas (p. ej. lepra, tuberculosis, etc.). A ello hay que añadir la incertidumbre que pueden suponer las modificaciones ambientales provocadas por el cambio climático en la producción y estabilidad de ecosistemas agrícolas, así como la capacidad de promover cambios en la dinámica poblacional de estas enfermedades (Graniti *et al*. 2011, Ponti *et al*. 2014, Martelli *et al*. 2016).

Aunque los nematodos fitoparásitos (NF) están ampliamente distribuidos en todas las zonas olivareras del mundo, el complejo de enfermedades que causa el parasitismo de NF en el cultivo del olivo no suele ser considerado como un problema sanitario de relevancia. Para ilustrar esta circunstancia solo basta repasar los principales tratados dedicados a este cultivo; en este sentido, la falta de consideración sobre estos organismos en los capítulos destinados a la sanidad vegetal es un aspecto contrastado, donde en algunos casos, ni siquiera son mencionados. Esta circunstancia está fuertemente influenciada y subyace de la falta de dedicación sobre este tema en la mayoría de los foros de formación académica y de divulgación científica sobre la sanidad del olivar. Aunque el grado de ausencia de estos agentes fitopatógenos en los planes fitosanitarios elaborados para el olivar por la Administración es cada vez menor, con frecuencia ocupan un lugar muy postergado entre las prioridades establecidas a la hora de fomentar educación, investigación y desarrollo científico.

Son diversas las razones por las cuales los NF como agentes fitopatógenos deben de considerarse un factor determinante en el diseño de los nuevos retos y perspectivas en la Fitopatología como disciplina científica y académica en el olivo. En este contexto, Nico (2012) señaló los posibles motivos que determinan el potencial que tienen los NF para constituir un problema fitosanitario relevante dentro del panorama oleico andaluz. Asimismo, en la misma tesis doctoral se enunciaron las posibles razones asociadas con el poco interés que subyace del problema sanitario que pueden causar estos organismos en dicho cultivo. No obstante, la importante transformación en el sector del olivar unido a los significativos avances en la nematología podría resultar en un nuevo enfoque temporal en el cual no se mantengan vigentes ciertas condiciones que determinan dicha problemática. Sin embargo, una adecuada revisión de las potenciales causas subyacentes a la baja relevancia que presenta actualmente el parasitismo de nematodos sobre olivo sería necesaria para una adecuada concienciación en el panorama actual.

Del mismo modo que el conocimiento de la biodiversidad es esencial para mantener el bienestar humano, desentrañar la diversidad de organismos potencialmente causantes de problemas fitosanitarios en un contexto agrícola es una de las claves del éxito que puedan tener las posibles medidas de control a efectuar (van der Putten *et al.* 2006). En este sentido, en los últimos años varios estudios han sido enfocados al descifrado de la diversidad de NF infestando suelos en importantes zonas olivareras de la cuenca Mediterránea (p. ej. Marruecos (Ali *et al.* 2017)). Sin embargo, en una de las zonas olivareras más importantes a nivel mundial, como es la ocupada en Andalucía (España), la falta de información al respecto es significativa. A pesar de algunos estudios realizados en Jaén (Peña-Santiago 1990) y en 79 parcelas distribuidas por Andalucía (Palomares-Rius *et al*. 2015), es notoria la escasez de estudios sistemáticos destinados a determinar la diversidad de estos organismos asociada a esta zona olivarera de elevada importancia tanto social como económica. Esta circunstancia suscita en cierto modo la necesidad urgente de realizar un estudio sistemático que describa la diversidad, así como la distribución de NF en el cultivo del olivo en Andalucía. Esto proporcionará un aumento en el conocimiento sobre los procesos ecológicos que regulan las poblaciones de estos organismos fitopatógenos, lo que proporcionará

un desarrollo de estrategias sostenibles y eficaces en el control de estos parásitos del cultivo del olivo.

1.1 Generalidades sobre la biodiversidad en los ecosistemas

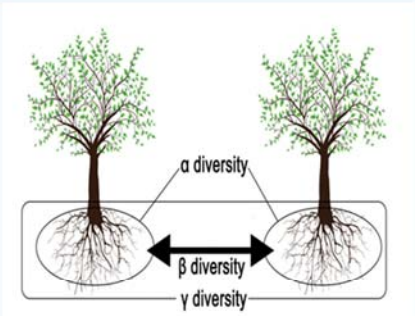
El término biodiversidad (o diversidad biológica) es un concepto fundamental, complejo y general, que abarca y está incluido en un amplio espectro de actividades de la ciencia y sociedad. Hacer una breve referencia sobre sus aspectos generales y su relación con la Agricultura, es esencial para una mejor comprensión del estado del arte y contexto de los objetivos planteados y metodología llevada a cabo en la presente Tesis Doctoral.

1.1.1 El concepto y la medida

Aunque la percepción de la variedad de formas de vida es tan antigua como la propia autoconciencia de la especie humana, el concepto de biodiversidad es relativamente reciente (Wilson 1988). Sin embargo, la biodiversidad se ha convertido en un término comúnmente utilizado en diferentes ámbitos de la vida pública (p. ej. científico, político, social, etc.). El concepto de biodiversidad puede ser intuitivamente simple de entender, pero es mucho más complejo de definir rigurosamente. A efectos prácticos, la biodiversidad puede considerarse como sinónimo de diversidad biológica. Desde que fue concebido por primera vez por Walter G. Rosen en 1986 mientras planeaba un foro sobre diversidad biológica (Foro Nacional sobre Biodiversidad en Washington, EEUU) hasta la fecha, el concepto de biodiversidad ha sido definido en numerosas ocasiones y usado en una variedad amplia de contextos (Harper y Hawksworth 1994, Gaston 2009). En definitiva, la biodiversidad es una expresión de la "variedad (o variabilidad) de la vida" y debe ser tratada como un recurso global, para ser indexada, usada, y sobre todo, preservada (Wilson 1988).

En el ámbito donde el concepto de biodiversidad puede hacerse más riguroso, conciso y con un mayor valor práctico es aquel donde se determinan las diferentes formas en que puede medirse (Gaston 1994). Podría decirse que el progreso reciente más importante en el estudio de la biodiversidad en el ámbito científico ha sido, por lo tanto relacionado con el desarrollo de tales medidas (Purvis y Hector 2000, Gaston 2009, Anderson *et al.* 2011, Baselga y Chao 2017). Tal desarrollo ha sido influenciado a su vez por los avances sustanciales en el campo de la biología molecular, concretamente en el desarrollo de nuevas técnicas de análisis genético de poblaciones (Purvis y Hector 2000). Una de las propiedades clave de la biodiversidad es que no se distribuye homogéneamente en el espacio, es decir, diferentes localizaciones generalmente albergan diferentes comunidades biológicas (Rosindell *et al.* 2011, Wiegand y Moloney 2014). Por lo tanto, la cuantificación de dichas diferencias es un paso importante para comprender cómo y por qué la biodiversidad se distribuye tal como es. En este sentido, tales medidas que determinan la diversidad de especies en una región pueden expresarse y se manifiestan en un rango de escalas (Whittaker 1972) (Recuadro 1). De una manera simplificada se puede decir que la diversidad local se conoce como ***diversidad alfa***, la diversidad total de una región como ***diversidad gamma***, y finalmente, la ***diversidad beta*** se define como la relación entre las diversidades gamma (regional) y alfa (local) (Whittaker 1960). En otras palabras, la diversidad beta cuantifica el número de comunidades diferentes en la región, y por lo tanto, es claro que dicha medida no solo explica la relación entre la diversidad local y regional, sino que también informa sobre el grado de diferenciación entre las comunidades biológicas (Tuomisto 2010). Otra forma de ver a la diversidad beta es como un término que refiere a la heterogeneidad en la composición de especies ya sea en el espacio o en el tiempo.

RECUADRO 1. Índices de medida de la biodiversidad



- alfa (α): es la diversidad intrínseca de cada comunidad de especies concreta del paisaje en cuestión

- beta (β): es la tasa de cambio en especies de dos comunidades adyacentes ya que entre dos comunidades de especies dinstintas geográficamente contiguas en el territorio, exisitiran diferencias y muy probablemetne especies comunes. Por tanto, refleja la diferencia de composición de las dos comunidades y en última instancia la heterogeneidad del paisaje.

- gamma (Υ): es la diversidad intrínseca de un paisaje, e integra los componentes alfa y beta de la diversidad. Estima la variedad de especies en una zona determinada, incluyendo todas las comunidades que se encuentran en ella.

Adaptada de Whittaker 1960, 1972

Una de las principales diferencias en el uso de las distintas variables descriptivas de la diversidad biológica (alfa, gamma y beta; Recuadro 1) subyace en el planteamiento de hipótesis con el fin de conocer y comprender los procesos biológicos que describen la variación de comunidades de especies (Whittaker 1960). En comparación con la diversidad beta, los estudios planteados con el uso de índices que abarcan la diversidad alfa y/o gamma no permiten probar hipótesis sobre dichos procesos ya que ignoran la identidad de las especies y el grado de diferenciación entre las comunidades biológicas (Tuomisto 2010). Esto se debe a que la diversidad alfa y gamma son diferentes si (y solo sí) las comunidades biológicas dentro de la región son diferentes. En definitiva, **la diversidad beta proporciona un conocimiento cuantitativo que vincula los procesos locales y regionales que dan forma a los patrones de diversidad de especies** (Whittaker 1960, Legendre *et al.* 2005, Anderson *et al.* 2006, Baselga 2013). El estudio de la diversidad beta en un

ecosistema puede ser muy útil para responder a cuestiones ecológicas que no podrían ser resueltas con el uso de las otras variables descriptivas de la diversidad.

1.1.2 Los factores que subyacen de su variabilidad

El estudio y comprensión de los factores que determinan los patrones de biodiversidad es uno de los desafíos centrales y más antiguos de la ecología de ecosistemas desde su aparición como su disciplina científica (Preston 1948). La estabilidad y estructura, así como la variación espacial de las comunidades de especies se rige por la propia estabilidad del entorno abiótico, las interacciones entre los componentes bióticos (incluido el huésped en el caso de parásitos), y por la solidez y el equilibrio de la propia comunidad (Vellend 2010). El establecimiento de prácticas agrícolas en ecosistemas naturales produce un régimen de perturbaciones ecológicas que influyen de manera determinista la diversidad de las comunidades de especies. Es decir, los factores que potencialmente pueden estructurar los patrones de biodiversidad (p.ej. propiedades del suelo, interacciones biológicas, etc.) están fuertemente afectados por las alteraciones ambientales ocasionadas por el efecto intrínseco de la agricultura (Leps y Rejmánek 1991, Zermeño-Hernández *et al.* 2015). Los regímenes de perturbaciones en los agroecosistemas están influenciados de manera gradual a su vez por el efecto de establecer diferentes prácticas agrícolas (p.ej. manejo del suelo, sistema de riego, cubiertas vegetales, etc.) en función del tipo y grado de explotación (José-María *et al.* 2010). En definitiva, las diferentes prácticas agrícolas que se apliquen en un agroecosistema deben ser consideraras por igual como procesos potenciales de estructurar los patrones de comunidades de especies (p.ej. organismos parásitos o beneficiosos) (Vellend *et al.* 2007).

Además de estos procesos deterministas que conforman el "nicho ecológico", los patrones de biodiversidad pueden además estar influenciados por procesos neutrales relacionados con las fluctuaciones aleatorias en la abundancia de especies, la dispersión limitada, o la biogeografía aleatoria (Vellend 2010). Estos procesos estocásticos están relacionados con "la Teoría Unificada Neutral de la Biodiversidad y

Biogeografía" que es lo opuesto a la teoría del nicho ecológico, es decir, todas las especies son idénticas y, ejercen y se someten a competencias iguales en su hábitat (Hubbell 2001). Hoy en día existe un consenso amplio de la coexistencia de ambos tipos de procesos (p.ej. deterministas y estocásticos) en dar forma y estructura a la variación espacial de las comunidades biológicas (Chave 2004, Adler *et al.* 2007). Por tanto, la determinación de la contribución relativa de los factores relacionados con el nicho ecológico y la teoría neutral en el ensamblaje de especies es un reto actual (Chase y Myers 2011). En los últimos años se ha avanzado notablemente en este sentido, desarrollándose numerosas herramientas y modelos que permiten explorar estas cuestiones, pero es importante entender la lógica de lo que se esté evaluando, así como el mecanismo subyacente. Con el fin de evitar resultados que pueden llevar a interpretaciones erróneas hay que tener en cuenta varios aspectos. En primer lugar, es fundamental tener claro la finalidad del estudio para elegir el modelo adecuado y en segundo lugar, es necesaria la adecuada selección de las herramientas adecuadas a utilizar (Alberti *et al.* 2018).

Una de las herramientas más adecuadas para explorar la importancia relativa entre los procesos estocásticos y deterministas en los ensamblajes de especies es mediante el uso de la diversidad beta. Dicha virtud radica en que en los patrones de variación en la composición y abundancia en dichos ensamblajes de especies difieren entre la teoría de nichos y neutral (Soininen *et al.* 2007, Vellend 2010, Chase y Myers 2011, Vellend *et al.* 2014, Kraft *et al.* 2015). De hecho, existe una amplia variedad de ambientes y de sistemas donde este enfoque ha sido aplicado con el fin de responder a una amplia gama de preguntas que van desde mecanismos de ensamblaje comunitario en bosques tropicales (Legendre *et al.* 2009, Myers *et al.* 2013) hasta la variabilidad espacial en comunidades de aves (Stegen *et al.* 2013), pasando por el efecto de la agricultura (Vellend *et al.* 2007), y los mecanismos que determinan la variación espacial de comunidades de organismos de suelo (Dumbrell *et al.* 2009). Sin duda, se ha avanzado mucho en los últimos años en determinar los factores que promueven la heterogeneidad en las comunidades des especies. Existen muchas aproximaciones para evaluar la importancia relativa de diferentes tipos de factores en el ensamblaje de especies, como, por ejemplo, *la influencia relativa del manejo agrícola y los factores ambientales en la*

estructura y distribución de comunidades de organismos parásitos de plantas. Como puede ser el caso de los NF de plantas, los organismos en los cuales se centra el presente trabajo.

1.1.3 Importancia y papel en la agricultura

La biodiversidad es crucial para la humanidad ya que es necesaria para mantener funciones y procesos claves de los ecosistemas, así como su estructura (MEA 2005). Además, su valor se ha vuelto más perceptible para la sociedad dado que la pérdida de ésta se relaciona con efectos perjudiciales en servicios ecosistémicos fundamentales (Barnes *et al.* 2018). Los beneficios ambientales suponen además beneficios sociales y económicos. Por ejemplo, **la biodiversidad es el fundamento de la agricultura ya que la gestión de ésta para mantener y/o mejorar las funciones de los ecosistemas es esencial para la optimización de la producción agrícola** (Gardner y Campbell 1992, Keesing *et al.* 2010). No obstante, existe la evidencia que a medida que se intensifican los sistemas de producción agrícola y su aislamiento con el fin de aumentar los rendimientos, estos sistemas tienden a perder su diversidad biológica además de su equilibrio, aumentando de este modo los brotes de los patógenos (Pimentel 1961, Keesing *et al.* 2010, Pagán *et al.* 2012). Por lo tanto, en una actividad de elevada importancia socioeconómica como es la agricultura, la biodiversidad puede ser valorada de diferentes formas resultando en un marco de beneficio positivo o negativo para dicha actividad (Mouysset *et al.* 2015). Desde una perspectiva ecológica, ásta puede ser clasificada en diversos componentes en función del papel que jueguen en el funcionamiento del sistema de producción agrícola (Altieri 1999). De todas las clasificaciones citadas hasta la fecha en la literatura, la que tiene una mayor aceptación por la comunidad científica es aquella que agrupa la **biodiversidad agrícola** en los siguientes grupos de organismos: **biota** que ofrece directamente recursos al ecosistema agrícola, **biota productiva** y **destructiva** (Swift y Anderson 1994). La presencia de algunas especies es beneficiosa para el ecosistema ya que proporcionan servicios ecosistémicos tales como la biodegradación de desechos, disponibilidad de nutrientes, o promueven la mineralización entre otros procesos biológicos fundamentales (Swift y Anderson 1994, Swift *et al.*

2004, Gresshoff *et al.* 2015). Además, otros organismos ejercen como antagonistas de plagas y/o enfermedades que puedan atacar a los cultivos reduciendo así la entrada de productos fitosanitarios, y por tanto, conservando la biodiversidad (Clergue *et al.* 2009). Por otro lado, otras especies presentan un valor positivo ya que desarrollan procesos con un beneficio directo, ya sea mediante bienes comercializables y/o materia prima para generación de productos agrícolas (Altieri 1999). Finalmente, el tercer grupo corresponde con el componente de la biodiversidad agrícola donde se encuentran las especies que producen una merma en la producción agrícola, como son los organismos patógenos y parásitos (Swift y Anderson 1994). Aunque a partir de lo referido anteriormente es posible agrupar la diversidad biológica presente en un ecosistema agrícola en diferentes componentes, es imposible señalar que especies presentan una mayor importancia que otras y por tanto, una forma objetiva de asignar un valor a la biodiversidad (Altieri 1999). La clave es identificar qué tipo de biodiversidad es deseable mantener y/o mejorar para llegar a cabo los servicios ecosistémicos, y luego determinar las mejores prácticas que fomenten los componentes de biodiversidad deseados (Swift *et al.* 2004).

A lo largo de la historia ha existido un extenso debate por la comunidad científica en cuanto a la conservación o no de la diversidad biológica correspondiente a los parásitos (Ingram 1999, Gómez y Nichols 2013). Sin embargo, resulta innegable que este grupo de organismos forman un componente de gran importancia en términos de diversidad biológica. Además son un elemento clave por sí mismo para la comprensión de como las comunidades biológicas están estructuradas, factor indispensable para el uso sostenible de los recursos bióticos (Dobson y Hudson 1986, Frainer *et al.* 2018). En un contexto fitopatológico, el estudio exhaustivo de la diversidad de parásitos y/o patógenos, así como señalar y entender los procesos que subyacen sobre el comportamiento y distribución de estos, es una información clave y necesaria para la toma de decisiones sobre su manejo sostenible para la conservación de los recursos naturales y para la producción agrícola (Ingram 1999, Swift *et al.* 2004). Por ejemplo, **conocer la diversidad presente de NF en un ecosistema agrícola es fundamental para el diseño y aplicación de adecuadas prácticas agrícolas para su manejo por diversos motivos: en primer lugar para comprender y descifrar**

los procesos ecológicos que determinan su distribución; y en segundo lugar, por la estrecha relación existente en algunas especies entre la especie de nematodo y la planta huésped (Freckman y Caswell 1985).

1.2 El suelo como fuente de biodiversidad

El suelo no es sólo una aglomeración de pequeñas partículas de materia orgánica y minerales, con iones que las plantas pueden usar. Es una entidad viva que hospeda innumerables organismos cuya diversidad puede incluso superar la de los organismos que viven fuera del suelo sobre la superficie terrestre. Dentro de este complejo hábitat, los nematodos son un grupo de organismos fundamental para el funcionamiento y desarrollo de los procesos que transcurren bajo el suelo y, por consiguiente, aquellos que ocurren sobre la superficie edáfica. Antes de centrarnos en estos organismos, y de manera especial en aquellos nematodos que se alimentan de plantas (NF), dada su complejidad e importancia dedicaremos una pequeña sección a resaltar aspectos generales sobre el suelo en un contexto relacionado con la biodiversidad del suelo y los sistemas agrícolas. Todo ello nos facilitará una mejor compresión sobre los procesos que determinan los patrones de diversidad de los NF en un agroecosistema, en este caso en el cultivo del olivo, componente y objetivo finalista de la presente Tesis Doctoral, el cual no podría entenderse si no se conocen ciertos aspectos generales del hábitat donde viven.

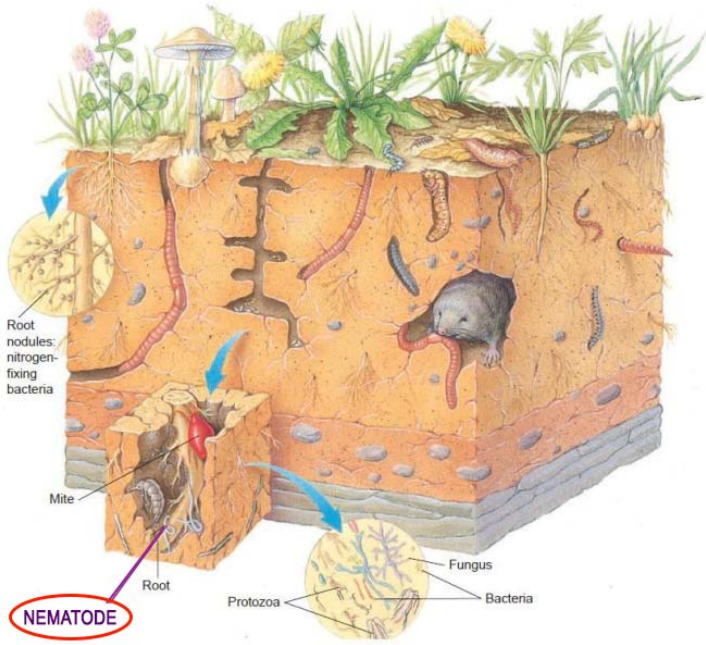


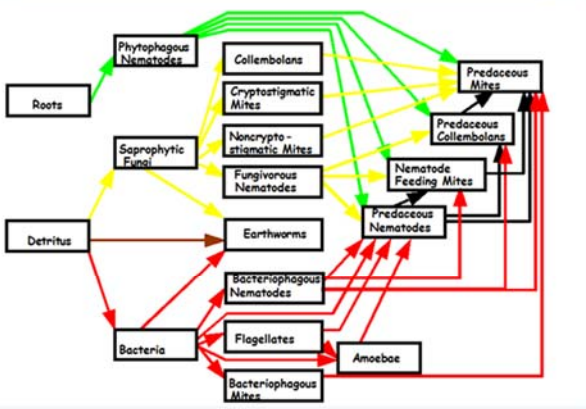
Figura 1.1: La biodiversidad del suelo (Fuente: Nemaplex)

1.2.1 Sistema diverso, organizado y desconocido

La biodiversidad del suelo está distribuida globalmente, desde los desiertos hasta las regiones polares pasando por los pastizales, bosques, las áreas urbanas y agrícolas (Wall *et al.* 2012). Los suelos albergan una cuarta parte de la biodiversidad de nuestro planeta y forman uno de los ecosistemas más complejos de la naturaleza, con infinidad de organismos que interactúan y contribuyen a los ciclos globales que hacen posible la vida (Figura 1.1). Probablemente los organismos de suelo representen el 25% de los 1.5 millones de especies descritas en todo el mundo (Decaëns *et al.* 2006). Es tal la diversidad que se estima en miles de especies por metro cuadrado presentes en la mayoría de los ecosistemas. En un contexto de espacio local, la riqueza de especies subterráneas es también mucho mayor que la encontrada en la vegetación o la correspondiente a la

fauna sobre el suelo (Decaëns 2010). En otras palabras, un solo gramo de suelo puede contener varios cientos de metros de hifas fúngicas y más de mil millones de células bacterianas, así como una amplia gama de nematodos, protozoos, lombrices, tardígrados, ácaros, moluscos, artrópodos, arácnidos entre otro tipo de fauna del suelo (Bardgett 2005, Wall *et al.* 2010, Bardgett y van der Putten 2014). Esta gran variedad de microrganismos y animales que viven en el suelo, interactuando entre sí y con las plantas y animales que viven sobre el suelo, constituyen una compleja red de actividad ecológica denominada red alimentaría del suelo (o **red trófica edáfica**) (Bardgett 2005). Esta red está constituida por una serie de niveles tróficos donde se producen una entramada red de interacciones dentro y entre cada uno de ellos a través de los grupos funcionales de organismos (Recuadro 2). Los grupos funcionales de organismos son asignados en función del tipo alimenticio de estos (p.ej. herbívoros, depredadores, fungívoros, bacteriófagos, etc.) (de Ruiter *et al.* 1995). Descripciones detalladas sobre los principales componentes de la red trófica edáfica están más allá del alcance y fundamentos de esta tesis doctoral (para más información y detalle sobre ello ver bibliografía específica (de Ruiter *et al.* 1995, de Vries *et al.* 2013, Paul 2014)). No obstante, esta red compleja y compuesta por una remarcable diversidad de organismos en continua interacción es responsable de procesos fundamentales de los ecosistemas tanto encima como bajo el suelo (Ferris *et al.* 2001, Wardle *et al.* 2004). Por ejemplo, procesos de tal importancia como: descomposición de la materia orgánica, ciclos bioquímicos, control de biomasa, ciclo del agua, descomposición y secuestro del carbono, modificación de la estructura del suelo, etc. (Ferris *et al.* 2001, Wardle *et al.* 2004, Wall *et al.* 2015). De hecho, se estima que la contribución de la biota del suelo a servicios ecosistémicos, como es la agricultura, es de 1.5 a 13 billones de dólares anuales (van der Putten *et al.* 2004).

RECUADRO 2. La red trófica del suelo



NEMATODOS EN LA RED TRÓFICA DEL SUELO

- Son los animales de suelo más diversos y abundantes
- Están presentes en cada nivel trófico

Fuente: Howard Ferris; NEMAPLEX (nemaplex.ucdavis.edu)

El grupo de organismos más numeroso y diverso incluido dentro de la red trófica del suelo es aquel formado por los microorganismos (p.ej. hongos y bacterias, también llamada microflora). No obstante, la abundancia y diversidad de la fauna del suelo es también extraordinaria, incomparable con otros ecosistemas sobre el suelo (Bardgett 2005). A lo largo de la historia han sido diversos los sistemas de clasificación desarrollados con el fin de obtener un mejor entendimiento del funcionamiento y estructura de la diversidad de este tipo de organismos del suelo (p.ej. por taxonomía, hábito alimenticio, etc.) (Bardgett 2005). Sin embargo, la información proporcionada por la clasificación taxonómica (p.ej. usando rangos jerárquicos) sobre el comportamiento de los estilos de vida y funciones de cada taxón dentro del complejo y diverso sistema del suelo ha sido poco relevante (Swift *et al.* 1979). Otra clasificación es la ordenación de los principales grupos taxonómicos en función del tamaño del cuerpo, siendo esta la más aceptada por la comunidad científica

(Figura 1.2; Swift *et al.* 1979, Decaëns 2010). De este modo, la fauna del suelo se estructuró en 4 grupos en función de la anchura del cuerpo: *microfauna* (menos de 0.1 mm), *mesofauna* (0.1-2 mm), *macrofauna* (2-20 mm) y *megafauna* (mayor de 20 mm). La anchura del cuerpo parece ser un criterio de clasificación más consistente que la longitud del cuerpo, ya que esta muestra una gran variabilidad incluso entre organismos del mismo grupo. Un ejemplo de ello lo encontramos en los nematodos que viven en el suelo, es tal la variabilidad de este grupo de organismos que podemos encontrar especies con una longitud 0.2 mm hasta especies que pueden superar el milímetro de longitud (Gaugler y Bilgrami 2004, Abolafia y Peña-Santiago 2016, Archidona-Yuste *et al.* Submitted to Contributions to Zoology). Sin embargo, los intervalos establecidos en esta clasificación no proporcionan limites estrictamente definidos, ya que en algunas conclusiones existe cierta confusión si un organismo en particular debe considerarse macro, meso o micro (Decaëns 2010).

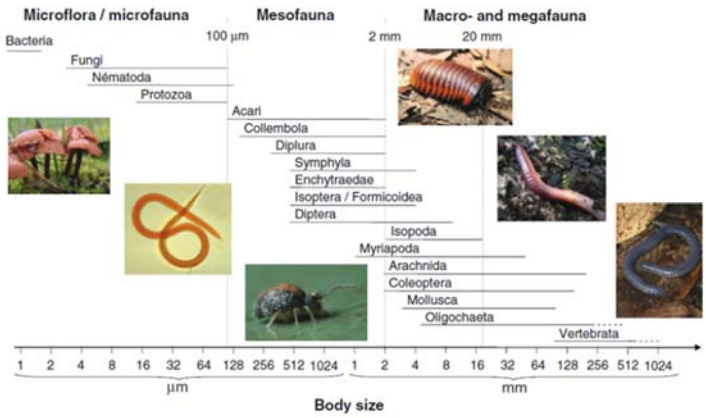


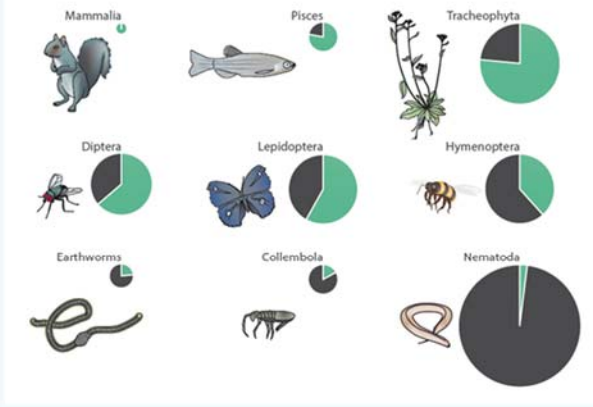
Figura 1.2: Clasificación de los principales grupos taxonómicos de organismos del suelo (Swift *et al*., 1979) (Fuente: Decaëns 2010).

A pesar de la importante contribución a la biodiversidad mundial, el conocimiento taxonómico de la biota del suelo sigue siendo pobre en comparación con la de la mayoría de organismos que viven sobre el suelo

(Decaëns *et al.* 2006, Phillips *et al.* 2017). Existen zonas donde dicha diferencia se ve incrementada, y donde la información sobre la biodiversidad de organismos de suelo es nula, lo cual impide realizar estudios macroecológicos que permitan obtener nuevos enfoques y conocimientos sobre los procesos edáficos (Cameron *et al.* 2018). Este déficit taxonómico para los organismos del suelo es de media del 76 %, y tiende a ser más alto para los taxones de cuerpo pequeño (Figura 1.2; Decaëns 2010). De hecho, uno de los grupos de organismos de suelo que junto con bacterias y hongos presentan un mayor déficit taxonómico son los invertebrados, siendo la falta de esfuerzo realizado en los nematodos como la posible razón de esta afirmación (Phillips *et al.* 2017). El porcentaje de especies descritas respecto al estimado dentro de los nematodos de suelo es extremadamente inferior respecto a otros organismos, ya que **solamente alrededor del 5 % de las especies estimadas han sido descritas** (Recuadro 3; Decaëns *et al.* 2006, Wall *et al.* 2012, Wall *et al.* 2015, Phillips *et al.* 2017). Pueden ser diversos los motivos que expliquen esta falta de información en este grupo concreto de organismos de suelo y en general, en la fauna que vive bajo el suelo. Aunque se están llevando a cabo progresos significativos para complementar esta falta de información (Ramirez *et al.* 2015, Phillips *et al.* 2017, André *et al.* 2002, Decaëns *et al.* 2006, Wilson *et al.* 2007, Decaëns 2010). Hechos como la escasez de expertos taxonómicos, la dificultad que lleva consigo describir especies de suelo o la presencia de *especies crípticas* (especies de características morfológicas y morfométricas prácticamente indiferenciables por métodos tradicionales, pero diferentes genéticamente) pueden estar uno de los principales motivos que estén detrás de esta tendencia. Este último aspecto queda reflejado claramente en los nematodos de suelo, donde la presencia de especies crípticas es notable, lo cual revela un número insospechado de este tipo de especies y dificulta de manera considerable la identificación de taxones mediante una base morfológica sin la ayuda de herramientas moleculares (Palomares-Rius *et al.* 2014b, Lee *et al.* 2017). En resumen, **seguir ignorando la inmensa diversidad biológica y la amplia gama de formas de vida que presenta el suelo, es un riesgo que no podemos permitirnos dada la importancia que subyace sobre el conocimiento de la biodiversidad bajo el suelo y su estrecha relación con la sostenibilidad de los**

ecosistemas y por consiguiente, el bienestar humano (van der Putten *et al.* 2004, Wall *et al.* 2015).

RECUADRO 3. La lista negra de la biodiversidad



Proporción de especies descritas (**VERDE**) y estimadas no descritas (**NEGRO**) para nueve taxones de especies que habitan sobre y bajo el suelo. Tamaño del círculo proporcional al número de especies estimadas.

NEMATODOS COMO ORGANISMOS CON LA MAYOR DE NECESIDAD DE ESFUERZO TAXONÓMICO

Fuente y adaptada de Phillips *et al.* 2017

1.2.2 Patrones de diversidad espacial en organismos de suelo

Una de las características más notables de la biodiversidad es que no se distribuye de manera homogénea (Bardgett *et al.* 2005, Wiegand *et al.* 2017). Los patrones espaciales de la diversidad de especies y los procesos asociados a los mismos han sido objeto de estudio desde hace mucho tiempo y desde diferentes escalas y enfoques, tales como la ecología de comunidades, biogeografía, agricultura, y más recientemente desde una perspectiva de la macroecología (Wiegand y Moloney 2014, Wiegand *et al.* 2017). La evidencia actual sugiere que algunos organismos de suelo

muestran patrones biogeográficos que son en cierto modo similares a los observados en aquellos organismos que viven sobre el suelo (Decaëns 2010, Soininen 2016), aunque los mecanismos que causan los patrones pueden ser diferentes (Wardle 2002). De hecho, se ha demostrado que los organismos de suelo no están distribuidos aleatoriamente, sino que están también condicionados por una estructura espacial al igual que aquellos que viven sobre el suelo (Ettema y Wardle 2002). Por otro lado, teniendo en cuenta la importancia que puedan tener los procesos estocásticos en los patrones de comunidades de organismos del suelo dada la complejidad de este (Wardle *et al.* 2004, Beck *et al.* 2015), la presencia de estructuras espaciales de la distribución de estos puede estar relacionada con gradientes ambientales (Ettema y Wardle 2002). Es interesante resaltar que el tipo y el grado de influencia de los procesos deterministas sobre la estructura espacial en la distribución de los organismos están determinados por la escala de estudio (Figura 1.3), que van desde milímetros hasta cientos de metros (Ettema y Wardle 2002). En la literatura podemos encontrar varios ejemplos que detallan el efecto de la escala de estudio sobre el tipo de procesos deterministas que estructuran la distribución espacial de organismos de suelo. Por ejemplo, Fromm et al. (1993) encontraron que, en un agroecosistema, la biomasa microbiana del suelo y de colémbolos era espacialmente dependientes en intervalos de más de 200 m, lo cual estaba relacionado con los gradientes de carbono del suelo a gran escala y las prácticas de cultivo. Por otro lado, Crist (1998) reveló que la distribución de termitas sigue patrones espaciales en un intervalo de hasta 330 m relacionándose con un gradiente topográfico y a tipos de vegetación. Del mismo modo la distribución de dos especies de nematodos parásitas de plantas (*Globodera rostochiensis* y *Heterodera avenae*) estaba subordinada a una patrón espacial a escala de campo de cultivo, lo cual estaba relacionado con el tipo de manejo del suelo efectuado en el ecosistema agrícola (Webster y Boag 1992). En cambio, en un estudio también realizado sobre NF se detectaron patrones micro-espaciales en la distribución de estos relacionándose esta vez con factores como la respiración del suelo (es decir, tipo de textura) y la presencia de la línea de cultivo (Delaville 1996). Estos ejemplos indican la importancia del control de los procesos deterministas en los patrones espaciales en la biota del suelo a diversas escalas. Sin embargo, dicha agregación espacial también está influenciada por procesos intrínsecos de la comunidad

(Figura 1.3), como pueden ser procesos estocásticos tales como la limitación de la dispersión y/o competencia entre individuos (Ettema y Wardle 2002, Vellend 2010). De hecho, son varios los estudios donde se deduce la importancia de los procesos estocásticos en la distribución espacial de nematodos, ya que la dependencia espacial no fue uniforme para toda la comunidad encontrada sino para ciertos géneros (Ettema *et al.* 1998), o un rango amplio (6-80 m) de la dependencia espacial de la distribución de nematodos también en un ecosistema agrícola (Robertson y Freckman 1995).

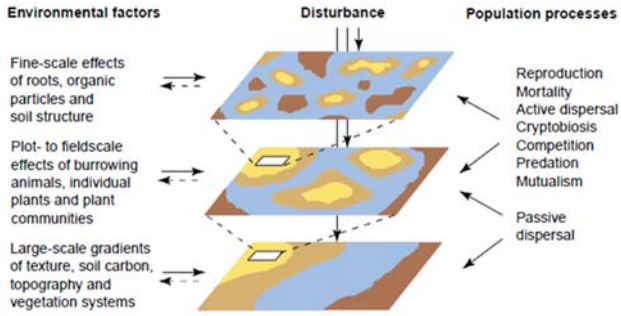


Figura 1.3: Heterogeneidad espacial de los procesos deterministas que estructuran la diversidad de los organismos de suelo (Fuente: Ettema y Wardle 2002)

Aunque hay un incremento considerable en la literatura de estudios enfocados a descifrar los procesos ambientales que subyacen de los patrones espaciales de organismos de suelo, la diferencia con los estudios realizados sobre el suelo es considerable (Barnes *et al.* 2018). Sin embargo, cada vez hay más evidencia que la ecología espacial del suelo puede arrojar nuevos conocimientos sobre la comprensión de los factores que mantienen y regulan la biodiversidad del suelo (Nielsen *et al.* 2011, Adhikari y Hartemink 2016, Durán *et al.* 2018), y cómo las distribuciones espaciales de los organismos de suelo influyen en el crecimiento de la planta y en la estructura de la comunidad vegetal, así como están relacionadas con el tipo de ecosistema y clima (Bardgett y van der Putten 2014, Nielsen *et al.* 2014, Wagg *et al.* 2014, Alsterberg *et al.* 2017). En definitiva, el hecho de que los datos para enfermedades en la agricultura se recopilan en una amplia variedad de niveles de información espacial

unido a la ya conocida heterogeneidad espacial de estos tipos de organismos de suelo (Perry 1996), refleja la importancia y las ventajas de la aplicación de esta metodología en un contexto fitopatológico ya que facilita el diseño de muestreo y/o el uso integrado de métodos de control eficientes sobre parásitos de plantas como son los NF (Porazinska *et al.* 2012, Godefroid *et al.* 2013).

1.3 Los nematodos, componente fundamental en el suelo

1.3.1 Generalidades

Los nematodos son organismos excepcionales. A pesar de su morfología engañosamente simple, y el hecho de tener un origen acuático, han tenido éxito en la colonización de una enorme gama de ecosistemas (Ferris *et al.* 2001, Yeates 2003, Gaugler y Bilgrami 2004). De hecho, son los invertebrados multicelulares más comunes y diversos que se conocen, ocupando todos los ecosistemas presentes en el planeta (Neher 2010). De hecho, cuatro de cinco animales multicelulares en el planeta son nematodos (Bongers y Ferris 1999). Aunque no hay duda de que los nematodos sean los metazoos más abundantes de la Tierra con una estimación conservadora de 10^{19} individuos (Lambshead 2004), existe cierta controversia sobre el número estimado de especies dentro del filo Nematoda. Tradicionalmente la estimación del índice de riqueza de especies se ha estimado principalmente en función de las características morfológicas y anatómicas complementadas con la función ecológica (Siddiqi 2000). Con la aparición y posterior notable avance de los marcadores moleculares, la incertidumbre sobre el número estimado de especies ha aumentado, ya que éste oscila en un rango que va desde un total de ~100.000 (Blaxter *et al.* 1998) hasta ~1.000.000 especies estimadas (Decaëns *et al.* 2006). Por otro lado, se estima que el número de taxones descritos está alrededor de 25.000 de especies de nematodos en todo el mundo, aunque no existen estudios recientes en este sentido (Chapman 2009). No obstante, si se compara el número de especies estimadas respecto al número que ya han sido descritas, el filo Nematoda es el grupo de animales que necesita el mayor esfuerzo taxonómico

(Decaëns *et al.* 2006, Chapman 2009, Decaëns 2010, Phillips *et al.* 2017), ya que la bibliografía indica que sólo entre 3-5% de las especies estimadas han sido descubiertas y descritas.

Independientemente de su hábitat, los nematodos tienen una morfología externamente simple, bilateralmente simétricos y no segmentados (Gaugler y Bilgrami 2004). No obstante, esta morfología básica se ve modificada en cierto grupo de especies, siendo en su mayoría nematodos parásitos de hábito sedentario (p.ej. *Meloidogyne* spp.). Los nematodos son animales lo suficientemente grandes (0.2-1.0 mm de longitud del cuerpo (Abolafia y Peña-Santiago 2016, Archidona-Yuste *et al.* Submitted to Contributions to Zoology) para ser identificados por microscopía óptica, y a su vez, lo suficientemente pequeños como para habitar las partículas de agua que rodean las partículas de suelo (Ferris *et al.* 2001), lo cual es imprescindible para una vida activa en estos organismos. A partir de esta necesidad primordial resulta otra peculiaridad singular pero a la vez sumamente importante, la asombrosa y amplia variedad de adaptaciones a condiciones de estrés ambiental (Gaugler y Bilgrami 2004, Treonis y Wall 2005). Aunque los nematodos son un grupo de organismos que demuestran una amplia variedad de adaptaciones a ambientes extremos de suelo y planta tales como la latencia desarrollada y diapausa en huevos en algunas especies (p.ej. *Heterodera* spp., *Globodera* spp.) (Subbotin *et al.* 2010), o el cambio de proporción de sexos para aumentar la probabilidad de sobrevivir en próximas generaciones en algunos casos (p.ej. *Meloidogyne* spp., la familia Longidoridae) (Papadopoulou y Traintaphyllou 1982), estos organismos son capaces de desarrollar respuestas fisiológicas de manera directa e inmediata a condiciones adversas (Bird y Bird 1991a, Gaugler y Bilgrami 2004). Por ejemplo, estas respuestas dinámicas incluyen la formación de larvas del estadio “dauer” (etapa alternativa adaptada para su supervivencia) relativamente resistentes, o el estado de quiescencia temporal tales como la anhidrobiosis (estado de desecación) entre otros (McSorley 2003). Otra característica resaltable es que los nematodos están caracterizados por la presencia de cuatro etapas de desarrollo pre-adultas, separadas entre ellas por la presencia de un estado de muda (Bird y Bird 1991b). Además de que, obviamente, son necesarias para el adecuado desarrollo del nematodo, estas etapas de crecimiento extremadamente importantes en

ciertos grupos de nematodos debido a su estrecha relación con actividades específicas (p.ej. migración a través del suelo, invasión e infección de huésped, procesos alimenticios, tasa metabólica, etc.) (Yeates y Boag 2003). Por otro lado, la existencia de estos estados de desarrollo también es clave para realizar una precisa identificación taxonómica de las especies de ciertas familias (p.ej. Longidoridae) (Loof y Luc 1990, Chen *et al.* 1997, Peneva *et al.* 2013, Tzortzakakis *et al.* 2016a).

Además de las especies parásitas de plantas o animales (nematodos parásitos, es decir, especies que dependen de un huésped), hay especies de nematodos que se alimentan de bacterias, hongos o simplemente son omnívoras, así como depredadoras; todos ellos conocidos también como nematodos de vida libre ("free-living nematodes") (Yeates *et al.* 1993). Además de la clasificación por hábito alimenticio, existen otros métodos que permiten estructurar la comunidad de especies en función de características ecológicas relacionadas con la capacidad de colonización y sucesión en un ecosistema después de una colonización (p.ej. escala "cp") (Bongers 1990, Bongers y Bongers 1998). Esta amplia variabilidad de atributos complementando con su extraordinaria diversidad y abundancia en el suelo, permite a los nematodos a ocupar la mayoría de los diferentes eslabones que componen la red trófica edáfica (Recuadro 2), influyendo directamente e indirectamente sobre los procesos ecológicos que acontecen en dicha cadena (Freckman y Caswell 1985). Por ejemplo, la contribución más importante de los nematodos en el ecosistema es la distribución de los nutrientes y minerales, **siendo responsables de un 30% de la mineralización del nitrógeno en el suelo**. Otras funciones que desempeñan estos organismos en las redes tróficas del suelo están relacionadas con la de regular las poblaciones de organismos oportunistas a través de la depredación, servir como presas de depredadores en niveles tróficos más altos, degradar toxinas que ingresen al ambiente, influir en la composición de la comunidad vegetal y su sucesión y acelerar las tasas de descomposición (Ferris 2010, Tsiafouli *et al.* 2017).

Todos los atributos citados convierten a los nematodos edáficos en un grupo de invertebrados de elevada importancia ecológica y económica no sólo por su efecto negativo en la producción agrícola (Neher 2010), sino por ser herramientas valiosas como indicadores del estado de

conservación del suelo tanto en sistemas agrícolas como naturales (Bongers y Ferris 1999, Ferris *et al.* 2001, Neher 2001, Yeates 2003). Son multitud de estudios los que avalan el uso de los nematodos (es decir, ensamblaje de las comunidades) como bioindicadores de una amplia variedad de procesos biológicos, ecológicos y agronómicos tales como la contaminación, procesos de sucesión de estados ecosistémicos, desertificación, o como reflejo del uso de diferentes prácticas agrícolas (Rossouw *et al.* 2008, Wilson y Kakouli-Duarte 2009, Zhang *et al.* 2015, Zhang *et al.* 2017). En este sentido, hay estudios recientes que evalúan las comunidades de nematodos así como sus ensamblajes en función de la aplicación prolongada de diferentes comunidades de nematodos en función de la aplicación de diferentes prácticas agrícolas a largo plazo en sistemas herbáceos (Zhang *et al.* 2017) o en plantaciones de frutales como es el caso del olivo cultivado (Sánchez-Moreno *et al.* 2015).

Después de las consideraciones anteriores, son diversas las causas que justifican el hecho de que los nematodos sean posiblemente el taxón del suelo con un mayor esfuerzo científico, aunque es reflejado en gran parte por el impacto económico directo producido por los NF sobre los cultivos. Sin embargo, aunque el interés de esta Tesis Doctoral se centre en los nematodos que parasitan las plantas y el cultivo del olivo como escenario, es interesante destacar que la mayoría de las especies que se encuentran en el suelo (alrededor del 70%) no se alimentan directamente de las plantas, pero pueden influir directa o indirectamente sobre la biología y ecología de aquellos. Además, si nos preguntamos si los nematodos no parásitos (es decir, especies de vida libre) pueden afectar al rendimiento de los cultivos, la respuesta es que sí, aunque indirectamente; por ejemplo, alimentándose de microflora simbiótica o de otros patógenos de plantas (Freckman y Caswell 1985, Gebremikael *et al.* 2016).

1.3.2 Los nematodos fitoparásitos

1.3.2.1 Relevancia, sintomatología, morfología y diversidad

El papel de los NF en el ecosistema del suelo hay que considerarlo desde dos vertientes. En primer lugar desde el punto de vista de su contribución a la génesis del suelo, ya que pueden contribuir a descomponer la materia

orgánica, la mezcla de esta con la parte mineral, aireación del suelo e incluso indirectamente a un aumento de la mineralización del nitrógeno del suelo, así como la concentración de N, P y K en la biomasa de las plantas (Tu *et al.* 2003, Perry y Moens 2006, Gebremikael *et al.* 2016). Sin embargo, el aspecto al que se ha prestado más atención, no sólo por la sociedad científica, está relacionado con sus atributos para producir efectos negativos sobre las funciones de los ecosistemas ya que pueden disminuir la productividad primaria alimentándose de las plantas (Ferris y Bongers 2006, Neher 2010). En un ecosistema agrícola, los efectos negativos por parte de los NF se ven incrementados, no sólo por la merma en la producción del cultivo a causa de la alimentación directa (ya sea por su ataque en las raíces, tallos, bulbos, parte aérea, fruto, etc.) sino también por las posibles infecciones microbianas o víricas que puedan facilitar, y las complejas interacciones entre ellas (Perry y Moens 2006). De hecho, la presencia de especies capaces de transmitir virus a la planta mediante su alimentación es un atributo a tener en cuenta (Taylor y Brown 1997). Dada la importancia fitopatológica que subyace de esta característica, en este documento se ha dedicado un apartado específico en este sentido donde los aspectos más relevantes serán expuestos. Por otro lado, se han descrito complejos de enfermedades donde los NF interactúan con otros patógenos (p.ej. *Fusarium* spp. o *Rhizoctonia solani*) incrementando la severidad y produciendo mayores pérdidas en los cultivos (Taylor 1990, Castillo *et al.* 1998, Castillo *et al.* 2003a, Björsell *et al.* 2017).

La magnitud de las pérdidas depende fundamentalmente de las densidades de población, de la susceptibilidad de la planta huésped, y de las condiciones ambientales (Scholthof 2006). Las pérdidas anuales en la producción en cultivos causada por los NF se estima en un rango desde 8.8-14.6% de la producción total de los cultivos lo que supone una pérdida económica de entre 100 a 157 billones de dólares a nivel mundial (Sasser y Krishnappa 1980, Koenning *et al.* 1999, Abad *et al.* 2008, Nicol *et al.* 2011). Otros estudios estiman estas pérdidas en un rango de 5.8-12.6% en ecosistemas destinados al pastoreo (Ingham y Detling 1984), y desde 1.4 hasta el 10% (Sohlenius *et al.* 1988) y entre 5-20% de la producción total en agroecosistemas (Singh *et al.* 2013). Es evidente que los estudios citados muestran un cierto grado de divergencia en los datos relacionado con el porcentaje estimado de pérdidas causadas por los NF. Este aspecto

puede estar relacionado con diversos factores tales como la falta de experiencia en el campo de la Nematología en ciertos países, presencia de interacciones complejas entre nematodos y otros organismos, efecto de la prohibición del uso del bromuro de metilo como fumigante, o la falta de rigor científico a la hora de anotar la pérdida generalizada en un campo agrícola (Taylor 2003, Zasada *et al.* 2010, Singh *et al.* 2013). No obstante, se han detectado grandes mermas en la producción agrícola por NF en importantes y variadas zonas agrícolas distribuidas por todo el mundo, llegando incluso a la pérdida completa en ciertos casos (Singh *et al.* 2013). Por ejemplo, en la cuenca mediterránea, las pérdidas de producción causadas por especies del género *Meloidogyne* en cultivos hortícolas se han estimado entre el 15 y el 60%. Concretamente la especie *Meloidogyne javanica* ocasionó mermas en la producción del 31% en el cultivo del calabacín lo cual supone una pérdida económica de 650.000€ anuales en los cultivos protegidos del sur de España (Talavera *et al.* 2012). Pérdidas similares e incluso mayores también ocasionadas por la misma especie se han observado en otros cultivos hortícolas en invernaderos, llegando hasta el 60% de pérdidas en el cultivo de tomate y pepino (Ornat *et al.* 1997). Análogamente, en campos agrícolas destinados al cultivo de frutales y cítricos se observaron pérdidas que oscilaban entre el 10 y el 33% (Sorribas *et al.* 2008).

La sintomatología que originan los NF en las plantas es inespecífica ya que el daño provocado por su parasitismo resulta poco aparente e incluso puede pasar desapercibido. En este sentido, no es extraño señalar que este grupo de organismos sea comúnmente conocidos con el sobrenombre de los "*enemigos ocultos de la agricultura*" (Palomares-Rius *et al.* 2014a, Palomares-Rius *et al.* 2016b). De hecho, no resulta fácil distinguirlos de otros problemas de tipo biótico o abiótico sin llevar a cabo un análisis nematológico que incluya un muestreo y análisis exhaustivo de suelo y raíces, el cual puede dificultarse si no se realiza con la ayuda de las indicaciones específicas facilitadas por parte de personal especializado o con la presencia de este. Los síntomas en la parte aérea de las plantas, como ya se ha indicado, son extremadamente similares a los producidos por otros patógenos de suelo o por aquellos ocasionados por cualquier estrés abiótico tal como la falta de nutrientes, falta de agua o la fitotoxicidad producida por la presencia de compuestos químicos (Castillo y

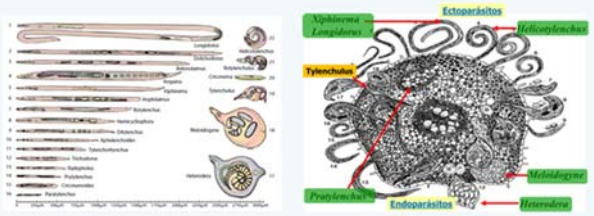
Verdejo-Lucas 2011). Por ejemplo, reducción del crecimiento de la planta en comparación con las aparentemente sanas, clorosis en mayor parte generalizada, declive o senescencia precoz. Aunque la reducción del crecimiento del sistema radical puede estar relacionada con otros organismos patógenos del suelo, los síntomas de las raíces de plantas infectadas por nematodos suelen ser más específicos. Síntomas como la presencia de deformaciones típicas tales como nódulos o agallas son atribuidos a especies del género *Meloidogyne*, o la aparición de un número alto de raíces secundarias puede atribuirse a especies pertenecientes a la familia Longidoridae. En definitiva, el daño que ocasionan los NF en el sistema radical dificulta la adecuada absorción de agua y nutrientes por parte de la planta lo que se traduce en anormal desarrollo de esta (Castillo *et al.* 2010). Por otro lado, es necesario destacar que a nivel de campo los problemas sanitarios en un cultivo ocasionados por NF se suelen manifestar con la presencia de rodales irregulares de crecimiento pobre, de forma variable pero que normalmente toma la forma circular.

Un aspecto reseñable de los NF que los hace diferencias del resto de nematodos que habitan el suelo es su notable variabilidad morfológica. Esta se debe a la variabilidad en la naturaleza de las alteraciones que causan estos a nivel citológico, histológico y fisiológico causada por su hábito alimenticio (Recuadro 4) (Seinhorst 1961). Por ello, los NF se suelen agrupar en: a) *ectoparásitos*, cuando el ciclo biológico se desarrolla complemente fuera de la planta; (especies migratorias del género *Xiphinema*, *Rotylenchus* o *Helicotylenchus* son encontradas en este grupo); b) *endoparásitos*, cuando su ciclo se desarrolla en todo o parte en el interior de la planta huésped. En este grupo podemos encontrar especies migratorias desplazándose dentro de la raíz (p.ej. *Pratylenchus* spp.) o en la parte aérea (*Ditylenchus dipsaci*), o bien sedentarios como las especies del género *Meloidogyne* o *Heterodera*; y c) un último grupo de especies *semiendoparásitas*, que intercambian un estado ectoparásito en la primera fase del ciclo vital y posteriormente, como sedentarios en el estado adulto. Especies como *Tylenchulus semipenetrans* o aquellas pertenecientes al género *Rotylenchulus* podemos encontrar en este último grupo (Siddiqi 2000). Por otro lado, una característica común en todos los NF es la presencia del estilete, estructura hueca a modo de aguja hipodérmica con la que penetran las células vegetales y con el que estas

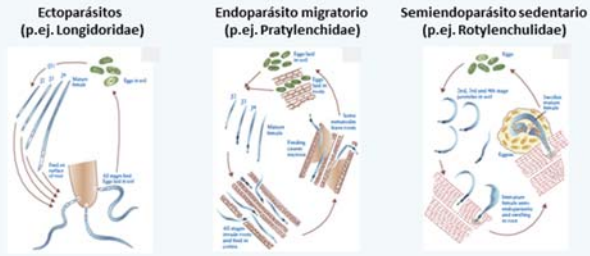
especies se alimentan succionando el contenido citoplasmático de las células vegetales (Gaugler y Bilgrami 2004). Entre los distintos géneros de este grupo de patógenos existe una elevada variabilidad en cuanto a su forma (Siddiqi 2000), al igual que de longitud, el cual puede oscilar entre 200 µm hasta más de 10 mm en especies pertenecientes a la familia Longidoridae, dentro de la cual también existe una amplia variedad en su longitud (Decraemer y Robbins 2007).

RECUADRO 4. Diversidad morfológica y de parasitismo

VARIABILDIAD MORFOLÓGICA Y DE PARASITISMO



DIVERSIDAD DE CICLO DE VIDA



Fuente y adaptada de Agrios 2005, Siddiqi 2000, y Bridge y Starr 2007

Resulta oportuno decir que al igual que los nematodos edáficos pueden encontrarse en cualquier ecosistema terrestre, cualquier planta (o cultivo) puede sufrir un perjuicio considerable como consecuencia del ataque por NF. En general, la mayoría de las especies parásitas de plantas exhiben una amplia gama de plantas hospedantes, forestales o agronómicas, así como anuales, bianuales o perennes (Perry y Moens

2006, Nicol *et al.* 2011). Sin embargo, la gama de huéspedes en algunos NF se reduce a una familia botánica (p.ej. *Heterodera avenae* parasita solo gramíneas o *Globodera* spp. solanáceas), y en otros casos se restringen sólo a un concreto grupo de especies (p.ej. *Tylenchulus semipenetrans* en cítricos y vid, o *Xiphinema index* en vid o higuera) (Castillo y Verdejo-Lucas 2011). Otro aspecto destacable es el notable aumento en los últimos años respecto a la descripción de nuevas especies mediante el uso de métodos moleculares, lo que ha llevado a más de 4.000 especies de nematodos a ser identificadas como NF lo que corresponde alrededor del 15% de las especies conocidas (Nicol *et al.* 2011, Palomares-Rius *et al.* 2017a, 2017b, 2018b, Archidona-Yuste *et al.* 2018). Sin embargo, sólo un pequeño grupo de especies pueden considerarse con elevada repercusión económica, ya sea por su efecto en el cultivo de manera directa o indirectamente mediante la transmisión de otros patógenos (Nicol *et al.* 2011). En este sentido, Jones y colaboradores (2013) publicaron una lista de las 10 especies de NF con una mayor relevancia científica y económica en todo el mundo donde podemos encontrar especies del género *Meloidogyne*, *Heterodera*, *Globodera* o especies concretas tales como *Xiphinema index*, *Ditylenchus dipsaci* o *Rotylenchulus reniformis* entre otras. Del mismo modo, el mismo estudio manifestó que los integrantes de dicha lista pueden sufrir cambios dependiendo de la zona de estudio y su repercusión económica (Jones *et al.* 2013). De hecho, cabe destacar que las pérdidas en la producción de los cultivos por NF podrían ser mucho mayores si las especies que actualmente producen daños en zonas localizadas si aumentaran su área de distribución causarían daños en zonas otras muy localizadas (Singh *et al.* 2013). Es decir, muchas especies tienen un impacto poco relevante en su zona nativa, pero este aumenta notablemente cuando se introduce en nuevas áreas. La entrada de *Bursaphelenchus xylophilus* (es decir, el nematodo del pino) en Europa es un claro ejemplo de ello, ya que se estima que las pérdida económicas ocasionadas desde su entrada en el viejo continente hasta los posteriores 22 años ascendieron hasta los 2 billones de Euros (Soliman *et al.* 2012). Visto el problema potencial desde un punto de vista sanitario y económico, la EPPO desarrolló una serie de estrategias internacionales con el fin de regular la expansión de especies con elevada importancia patogénica. Entre ellas se incluye las listas A1 y A2, donde podemos encontrar especies de NF de cuarentena en la Unión Europea, por lo que su

detección implica restricciones para la venta y exportación de material vegetal (Tabla 1.1) (Bello *et al.* 2005).

Tabla 1.1 Listas A1 y A2 de especies de nematodos de cuarentena.

Lista A1
Nacobbus aberrans
Radopholus similis attacking citrus (formerly R. citrophilus)
Xiphinema americanum sensu stricto
Xiphinema bricolense
Xiphinema californicum
Lista A2
Aphelenchoides besseyi
Bursaphelenchus xylophilus
Ditylenchus dipsaci
Globodera pallida
Globodera rostochiensis
Heterodera glycines
Meloidogyne chitwoodi
Meloidogyne enterolobii
Meloidogyne fallax
Meloidogyne mali
Radopholus similis (not attacking citrus)
Xiphinema rivesi

Fuente: EPPO Bulletin (https://www.eppo.int)

1.3.2.2 La identificación de los nematodos fitoparásitos

Tradicionalmente, la identificación taxonómica de estos organismos se ha basado exclusivamente en la comparación de características morfológicas, que se determinan cualitativa y cuantitativamente por técnicas de microscopía. La morfología de los nematodos puede ser caracterizada y simplificada a partir de una combinación de medidas y ratios derivados de diferentes partes del cuerpo (Jairajpuri y Ahmad 1992). Los caracteres e

índices más importantes provienen de los introducidos por de Man (de Man 1880), y deben su relevancia al hecho de que pueden ser utilizados para la identificación de cualquier género o especie de nematodo, ya sea parásito como de vida libre. No obstante, no hay que olvidar que cada grupo de nematodos presenta una singularidad que lo hace diferenciar del resto, siendo un carácter determinante a incluir en cualquier proceso de diagnóstico. En este sentido la presencia del estilete es un carácter diferenciador de los NF y además, es un elemento clave en el diagnóstico de estos organismos debido a su variabilidad en el tamaño y morfología existente entre familias, géneros y/o especies en este grupo de nematodos (Recuadro 5) (Siddiqi 2000, Perry y Moens 2006, Decraemer y Robbins 2007). Por consiguiente, en el diagnóstico de NF no sólo debemos considerar los índices universales citados antes (de Man 1880) sino que debemos incluir caracteres morfológicos determinantes a la hora de descifrar la nematofauna fitoparásita en estudio, como puede ser el estilete (Recuadro 5). En definitiva, cualquier clave taxonómica para la identificación de NF a nivel de género o especie requiere el uso conjunto de estos valores, por lo que en el proceso de diagnóstico es imprescindible un estudio morfológico preciso por parte de personal cualificado que incluya la toma de medidas mediante el sistema de microscopia adecuado. Existe una amplia gama de claves taxonómicas, desde aquellas destinadas a la identificación de familias y géneros, hasta el diagnóstico preciso de especies de grupos de NF de interés mediante el uso de herramientas dicotómicas y/o politómicas (Loof y Luc 1990, Loof y Chen 1999, Siddiqi 2000, Lamberti *et al.* 2004, Castillo y Vovlas 2005, 2007, Hunt y Handoo 2009).

RECUADRO 5. Caracteres morfológicos de diagnóstico

L = Longitud total del cuerpo del nematodo (µm).

L'= Longitud del cuerpo desde la región anterior del cuerpo hasta la abertura anal (µm).

a = Relación entre la longitud total del cuerpo y el diámetro máximo del cuerpo.

b = Relación entre la longitud total del del cuerpo y la longitud de la faringe.

b'= Relación entre la longitud total del del cuerpo y la distancia entre el extremo anterior del cuerpo y el extremo posterior de las glándulas faríngeas.

c = Relacionn entre la longitud del cuerpo y la longitud de la cola.

c'= Relación entre la longitud de la cola y el diámetro del cuerpo a nivel del ano.

V = Relación entre la distancia entre la región anterior del cuerpo hasta la vulva y la longitud del cuerpo (%).

V'= Relación entre la distancia entre la región anterior del cuerpo hasta la vulva y la longitud del cuerpo medida dese la región anteior hasta la abertura anal (%).

T = Relación entre la distancia entre la abertura de la cloaca hasta la parte anterior del testículo y la longitud del cuerpo (%).

m = Relación entre la distancia entre la parte cónica del estilete y la longitud total del estilete (%; Tylenchidos).

o = Relación entre la distancia entre la abertura de la gándula dorsal faríngea y la longitud del estilete (%).

MB = Relación entre la distancia entre la extremos anterior del cuerpo hasta el bulbo medio y lontigud del la faringe (%).

j = Longitud de la región hialina (µm).

Adaptada de Perry y Moens 2006)

Los nematodos son considerados como uno de los organismos que presentan una mayor dificultad a la hora de realizar una identificación taxonómica (Coomans 2000). Dicha dificultad subyace de numerosos factores que abarcan desde la elección del método más preciso y adaptado, pasando por la influencia de varios parámetros que pueden afectar al rendimiento de la identificación como el pequeño tamaño de estos organismos o la alta diversidad de especies presente en la muestra suelo, hasta la ausencia de características morfológicas especificas a causa de la similitud y coincidencia de la morfometría entre especies (Floyd *et al.* 2002, Chitwood 2003). La presencia de mezcla de especies estrechamente relacionadas en la muestra de suelo en estudio es una circunstancia habitual lo cual resulta en una mayor dificultad en el diagnóstico preciso dado que la discriminación entre especies se basa en

el promedio de las mediciones de la población de especímenes (Coomans 2002).

Con el fin de solventar los inconvenientes citados y facilitar de este modo el diagnóstico de los NF, la taxonómica descriptiva y tradicional es acompañada actualmente por el uso de la biología molecular. En este sentido, la evolución y desarrollo en este campo ha sido notable en las últimas décadas (Seesao *et al.* 2017). De hecho, no es nada nuevo decir que la mayoría de los progresos recientes en el diagnostico nematológico han sido en buena parte gracias al desarrollo de las técnicas moleculares como la reacción en cadena de la polimerasa (PCR) y bioquímicas basadas en la comparación de aminoácidos por electroforesis como es el caso de especies del género *Meloidogyne* (Powers 2004, Palomares-Rius *et al.* 2017b, Seesao *et al.* 2017, Archidona-Yuste *et al.* 2018). Todo ello ha resultado en un notable incremento en el diagnóstico y descripción de nuevas especies de NF, siendo actualmente el índice de riqueza mundial mayor que 4.000 especies (Gaugler y Bilgrami 2004, Nicol *et al.* 2011, Palomares-Rius *et al.* 2017a, 2017b, 2018b, Phillips *et al.* 2017, Archidona-Yuste *et al.* 2018). Además, es conveniente resaltar que la implantación de dichas técnicas ha aumentado la fiabilidad en la caracterización de especies, lo cual repercute positivamente disminuyendo la posibilidad de realizar identificaciones erróneas de NF y por lo tanto, en el diseño de las posibles estrategias de control (Perry y Moens 2006). Es decir, la gran cantidad de datos genéticos producidos mediante la biología molecular es una excelente materia prima para la identificación fiable de los NF.

A grandes rasgos, la identificación genómica se basa en la extracción de ADN del espécimen en estudio, la amplificación de un determinado fragmento de éste mediante la técnica de PCR y la caracterización de las diferencias interespecíficas de nucleótidos de estos fragmentos basado en la comparación con la información disponible en las bases de datos. En definitiva, las técnicas moleculares permiten la recolección precisa y rápida de grandes cantidades de datos que pueden ser utilizados por otros usuarios con el fin de realizar no sólo estudios de caracterización de especies, sino enfoques destinados a descifrar los procesos evolutivos y ecológicos característicos de las especies en estudio. Por ejemplo, dadas las limitaciones en la dispersión activa que caracterizan a los nematodos,

los estudios de la estructura génética de comunidades (es decir, patrones de variabilidad genética inter- e intrapoblacional) pueden arrojar nuevas ideas sobre los procesos evolutivos y ecológicos que determinan su distribución (Wiegand *et al.* 2017); así como incrementar el conocimiento sobre los procesos coevolutivos entre parásito y planta huésped, lo cual puede facilitar la toma de decisiones para el manejo y control de los NF (McDonald y Linde 2002, Plantard y Porte 2004). En este sentido, varios estudios han constatado el uso del ADN mitocondrial (ADNmt) como herramienta para el análisis de variabilidad genética en poblaciones de NF con el fin de descifrar los potenciales factores que determinan su distribución y han dirigido su evolución (Hugall *et al.* 1994, Blouin *et al.* 1998, Gutiérrez-Gutiérrez *et al.* 2011, Palomares-Rius *et al.* 2017a). Por otro lado, la aplicación de nuevas técnicas moleculares basadas en la secuenciación de regiones de ADN ribosómico altamente conservadas (el gen 18S, la región D2-D3 del gen 28S y la región ITS) ha facilitado la delimitación taxonómica de especies, y se ha mostrado como una excelente herramienta para el estudio de las relaciones filogenéticas en multitud de importantes grupos de NF (Ye *et al.* 2004, Oliveira *et al.* 2006, Cantalapiedra-Navarrete *et al.* 2013, Gutiérrez-Gutiérrez *et al.* 2013, Subbotin *et al.* 2014, Tzortzakakis *et al.* 2014, Araya *et al.* 2016, Archidona-Yuste *et al.* 2018, Palomares-Rius *et al.* 2018b, Singh *et al.* 2018). Además, estos marcadores moleculares han mostrado resultados interesantes sobre la estrecha relación entre huésped y endosimbionte revelando nuevas rutas para incrementar el conocimiento sobre los procesos de evolución de los NF (Palomares-Rius *et al.* 2016a).

La aplicación de los métodos moleculares en la identificación y caracterización de los NF han puesto de manifiesto la notable presencia de complejos de especies crípticas (Palomares-Rius *et al.* 2014b). Nótese el significado del concepto de especies crípticas, “dos o más especies distintas que están erróneamente clasificadas bajo el nombre de una especie” (Bickford *et al.* 2007). O lo que es lo mismo, especies que son morfológicamente muy parecidas llegando a ser casi idénticas pero que difieren genotípicamente. Actualmente existe cierta controversia en la sociedad científica sobre el origen, definición así como sobre los métodos utilizados para identificar especies crípticas, y cómo ello afecta a los estudios enfocados a los procesos de evolución de dichos complejos de

especies (Heethoff 2018, Struck *et al.* 2018a, 2018b). Una razón plausible del alto grado de especies crípticas en los NF fue reseñada por Coomans (2002), que señaló como principal causa la elevada diversidad de estos organismos presente en el suelo y a su vez, la posible divergencia en la celeridad entre la evolución morfológica y molecular como resultado de diferentes mecanismos de origen. No obstante, los marcadores moleculares de ADNr comentadas anteriormente así como el fragmento de ADNmt que codifica la proteína citocromo c oxidasa (*CO1*) han mostrado ser excelentes herramientas para la correcta caracterización y estudio de las relaciones filogenéticas de especies crípticas en NF (Madani *et al.* 2010, Palomares-Rius *et al.* 2014b).

En definitiva, los avances tecnológicos han mejorado la sensibilidad, la precisión y el rendimiento en el diagnóstico de los NF. Se dispone de varios enfoques, desde los métodos de identificación básica (morfológica y morfométrica) hasta las tecnologías de secuenciación de alto rendimiento más complejas (p.ej. "DNA barcoding") (Palomares-Rius *et al.* 2017b, Seesao *et al.* 2017). Aunque son numerosas las ventajas que ofrecen, el uso de herramientas moleculares en el diagnóstico de nematodos también posee importantes limitantes (p.ej. requisito de poseer material de referencia, elevado coste, difícil estandarización etc. (Seesao *et al.* 2017)). Estos limitantes pueden ser suprimidos con el uso aditivo de herramientas clásicas de diagnóstico, complementando ambos enfoques con el fin de realizar una taxonomía integrativa, la cual ha prestado fiables e importantes resultados en la caracterización de una amplia gama de NF (Cantalapiedra-Navarrete *et al.* 2013, Gutiérrez-Gutiérrez *et al.* 2013, Janssen *et al.* 2017, Archidona-Yuste *et al.* 2018). Además, la aplicación de un enfoque integrativo ha mostrado excelentes resultados en la identificación de especies crípticas, lo cual sería muy complicado con el uso exclusivo de alguno de los enfoques citados (Gutiérrez-Gutiérrez *et al.* 2010, Palomares-Rius *et al.* 2014b). Sin embargo, la presencia de personal con experiencia de taxonomía clásica es cada vez más escasa. Este hecho crea numerosos problemas en diversos campos de la nematología. En un contexto global, existe una demanda creciente de taxónomos clásicos de nematodos para evaluar la estructura de la comunidad en relación con la función del suelo (Wardle *et al.* 2004), y especialmente en aquellos NF de importancia económica en la agricultura (Ferris 1994, Coomans 2002,

Seesao *et al.* 2017) para el desarrollo posterior de herramientas eficientes de manejo y abordar las regulaciones de las especies de cuarentena (Powers 2004).

1.3.2.3 El ciclo vital y su relación con el control

A pesar de la diversidad y complejidad notable que exhiben los NF, el ciclo biológico presente en todas las especies sigue el mismo modelo básico. Es decir, el estado de huevo seguido de cuatros etapas de desarrollo juvenil y en último lugar, la fase adulta la cual puede desarrollarse en hembra o macho. No obstante, la duración del ciclo varía notablemente entre los diferentes géneros de NF, oscilando entre unos pocos días hasta casi un año en condiciones ambientales óptimas y en presencia de un huésped favorable (Gaugler y Bilgrami 2004). Asimismo, la existencia de diferentes hábitos alimenticios se manifiesta fundamental-mente en substanciales variaciones en un contexto biológico y funcional de las diferentes etapas de desarrollo del ciclo biológico básico del nematodo. Por ejemplo, la etapa de desarrollo atribuida como parásita y/o infectiva no coincide en todos los NF (Recuadro 4). Es decir, todos los estados de desarrollo (desde el juvenil de primera edad hasta los adultos) de especies ectoparásitas presentan la capacidad de parasitar a la planta huésped; en cambio, en los nematodos semiendoparásitos del género *Rotylenchulus*, la planta huésped sólo es parasitada, y en este caso infectada, por parte del estado de desarrollo adulto ya que el resto de estadios no se alimentan (Robinson *et al.* 1997, Decraemer y Robbins 2007). Además, las especies endoparásitas como las pertenecientes al género *Meloidogyne* y *Heterodera* solo presentan un estado de desarrollo capaz de infectar a la planta, y es el juvenil de segunda edad, aunque el resto de fases excepto el juvenil de primera edad son capaces de alimentarse (Perry y Moens 2006). Otro ejemplo de variabilidad entre grupos de especies está en la fase del huevo. El huevo es generalmente elipsoidal con una cubierta de espesor variable. La diferencia entre grupos de nematodos de diferente hábito alimenticio está en la forma en la que los huevos son depositados por la hembra, que puede ser individualmente (p.ej. Longidoridae, Trichodoridae, etc.) o en masa, manteniéndose dentro de una matriz gelatinosa (nematodos de las agallas, *Meloidogyne* spp.), o protegiéndose dentro de un quiste duro

formado a partir del cuerpo la hembra muerta (nematodo de los quistes, *Heterodera* spp. y *Globodera* spp.) (Siddiqi 2000, Coomans *et al.* 2001). Por otro lado, en la siguiente fase al proceso de eclosión también nos encontramos un ejemplo de variación entre grupos de especies, ya que el juvenil que nace del huevo en las familias Longidoridae y Trichodoridae es el de primera edad, mientras que en "Tylenchina" es el juvenil de segunda edad ya que la primera muda ocurre dentro del huevo (Siddiqi 2000, Coomans *et al.* 2001). Finalmente, se ha documentado una cierta variabilidad en la resistencia a estreses ambientales en las diferentes fases de desarrollo entre ciertos grupos de NF, siendo generalmente el estadio más resistente el juvenil de tercera edad (Perry *et al.* 2006).

En definitiva, la variabilidad biológica en el ciclo de vida de los NF añade aún más complejidad a la hora de intentar conocer los factores que determinan la variabilidad en las comunidades de estos organismos de suelo. Por lo tanto, es evidente que en la situación en la que tengamos un problema fitosanitario ocasionado por una mezcla de grupos de especies de NF, la dificultad en el diseño de estrategias de manejos eficientes se ve incrementada de manera considerable. Esto es porque un componente clave del control de la enfermedad no sólo está supeditado a un diagnóstico preciso sino también al conocimiento del ciclo de vida del patógeno en particular. De hecho, revelar el ciclo de vida de los patógenos de suelo, como pueden ser los NF, puede proporcionar un substancial conocimiento para herramientas potenciales para su manejo (Perry y Moens 2006). En la mayoría de enfermedades causadas por NF (especialmente aquellas que son causadas por semi- o endoparásitos), el ciclo de ésta se asemeja bastante al ciclo de vida de la especie que ejerce como patógeno. Por lo tanto, en la situación donde el problema fitosanitario esté causado fundamentalmente por sólo una especie, el conocimiento exhaustivo del ciclo del patógeno será determinante para el diseño de herramientas de manejo y control (Norton 1978). No obstante, las herramientas disponibles para una especie determinada pueden variar considerablemente si la especie patogénica es otra, especialmente cuando el ciclo de vida entre ellas presenta notables diferencias.

En general, el objetivo del control de los NF es evitar que se produzca una merma significativa en la producción del cultivo. Para tal fin existe una

amplia gama de herramientas basadas en métodos preventivos que tienden a evitar la entrada y establecimiento del patógeno (p.ej. acciones legislativas como la lista de cuarentena de especies), así como métodos paliativos con el fin de disminuir la densidad de población por debajo del umbral económico y/o reducir la tasa de multiplicación de la población del nematodo (p.ej. métodos culturales, químicos, mejora vegetal, etc.) (Castillo *et al.* 2010). No obstante, es necesario enfatizar que una vez establecido el problema no hay método de control que reduzca las pérdidas de producción por completo y/o que disminuya las densidades de población de la especie de nematodo patogénica en torno al 90% de forma consistente y prolongada en el tiempo (Perry y Moens 2006). A todo ello hay que añadir el descenso considerable en los últimos años de los métodos químicos, lo cuales han sido ampliamente usados por su elevada eficacia. Las restricciones legislativas y/o el aumento de la preocupación por parte del consumidor en un contexto sanitario y ambiental, han sido las causas del descenso de los fumigantes de suelo y nematicidas en el control de los NF (Taylor 2003, Zasada *et al.* 2010, Singh *et al.* 2013). No obstante, a lo largo de los años se ha demostrado que el control de NF mediante el uso exclusivo de una sola técnica (p.ej. métodos químicos) no tiene un efecto perdurable o este es parcialmente efectivo (Hildalgo-Diaz y Kerry 2008). De hecho, a lo largo del tiempo son varios los estudios que han resaltado que el control de los NF debe de realizarse mediante la implementación de variadas estrategias de manejo que combinen diferentes métodos (p.ej. culturales, biológicos, químicos, etc.) de manera compatible e integrada en el espacio y tiempo. Es decir, se ha constatado que la eficacia del uso integrado de varios métodos debe ser concordante y aditiva de manera que se produzca una secuencia en la aplicación, ya sea antes del establecimiento del cultivo en un contexto preventivo o durante este con el fin de evitar que se produzca el menor daño económico posible. Además, las prácticas seleccionadas e incluidas en el programa de manejo deben de tener un bajo impacto ambiental, y además, económicamente viables (Perry y Moens 2006, Castillo *et al.* 2010). Para finalizar es necesario volver a enfatizar que la selección de las estrategias de control debe de estar basada en el conocimiento de las características biológicas de las especies de NF que estén causando el problema fitosanitario, mediante el estudio previo de su ciclo de vida y de la enfermedad. Todo ello no sólo ayudará a la selección de dichas

herramientas, sino que permitirá establecer un programa de actuación donde se establecerán los momentos óptimos de intervención a partir de una adecuada predicción del potencial efecto que pueda tener sobre la viabilidad de la relación nematodo-planta tanto a corto como a largo plazo (Sorribas y Ornat 2011).

1.3.2.4 Distribución espacial de los nematodos fitoparásitos y su importancia en el control

Como ya se ha mencionado anteriormente, la estructura del soporte y distribución de la biodiversidad a menudo son el resultado de la influencia de un conjunto de elementos bióticos y abióticos (Adler *et al.* 2007). La influencia de estos procesos, especialmente en los organismos de suelo como es el caso de los NF, está estrechamente relacionada con la escala de estudio espacial (Ettema y Wardle 2002). En definitiva, la estabilidad de las comunidades de NF se rige por la estabilidad del entorno abiótico, las interacciones entre los componentes bióticos, incluido el huésped, así como la solidez y el equilibrio de la propia comunidad. Hay que recordar que además de estos procesos determinísticos, la distribución de patrones de comunidad en los NF puede estar también determinada por procesos estocásticos.

El patrón espacial de las poblaciones de NF en un ecosistema agrícola es una de las características ecológicas más exclusivas de estos organismos dadas sus implicaciones prácticas tales como su manejo y control (Yeates y Boag 2004, Been y Schomaker 2006). En general los NF tienen una distribución espacial agregada (Seinhorst 1982, Webster y Boag 1992, Ettema y Wardle 2002), que puede estar determinada potencialmente tanto por factores bióticos y abióticos responsables de su distribución local y regional. Entre estos factores deterministas se incluyen las características del suelo, ya sea su propiedades físicas y/o químicas, interacciones con la microbiota, la planta huésped, distribución y morfología de las raíces de la planta, prácticas de manejo agronómico, características climáticas, tipos de relación fisiológica con la planta (es decir, estrategias de alimentación y ciclo vital), así como la historia inicial de la introducción y de la parcela del cultivo (Norton 1978, 1989, Been y

Schomaker 2006, Neher 2010). No obstante, la influencia de estos factores puede variar según la escala de estudio (Figura 1.3) y pueden estar espacialmente estructurados (Legendre *et al.* 2009). Aunque la distribución espacial de nematodos se ha investigado para comparar los nematodos en diversos hábitats (p.ej. natural y agrícola (Freckman y Virginia 1989, Duyck *et al.* 2012)) o para analizar la distribución geográfica de una especie particular dada su importancia económica (Webster y Boag 1992) y grupos de especies (Liébanas *et al.* 2002, 2004, Archidona-Yuste *et al.* 2018), es escasa la información disponible sobre la distribución espacial de comunidades de NF en sistemas agrícolas.

En definitiva, aunque la importancia de conocer la biodiversidad de un cierto grupo de organismos en un ecosistema determinado es evidente (Bardgett y van der Putten 2014), lo es aún más conocer las razones de su distribución. La importancia de este aspecto ve incrementada su relevancia en el caso de organismos que presenten un impacto económico, como es el caso de los NF. En un ecosistema agrícola donde la presencia de NF puede llegar a ser un problema fitopatológico y por tanto económico, una de las primeras actuaciones que deben de llevarse a cabo es conocer los factores que determinan la variabilidad y distribución de las comunidades de éstos; información esencial para el correcto diseño de estrategias eficientes de manejo y control a nivel de campo (Been y Schomaker 2006). Sin embargo, es sorprendente la falta de estudios en este sentido, donde a partir de un muestreo sistemático realizado en un sistema agrícola se descifre la diversidad de NF y se determinen los factores que subyacen de los patrones espaciales de estas comunidades. En este sentido, el agroecosistema del olivar andaluz muestra una extensa variabilidad de factores bióticos y abióticos tales como una amplia diversidad de sistema de manejo agronómico, genotipos de olivo, tipos de suelo, variabilidad topográfica y agroclimática que lo hacen un escenario ideal para el estudio de la biodiversidad y los factores ecológicos que determinan su distribución (Cox *et al.* 2016).

1.4 El cultivo del olivo, un sistema agrícola ideal

1.4.1 Importancia del olivar en España y Andalucía

El cultivo del olivo (*Olea europaea* L. subsp. *europaea*), junto con la vid (*Vitis vinífera*), constituyen los sistemas agrícolas más arraigados en el paisaje español, y representan los dos grandes sistemas de producción frutal de secano en España (Rallo 1998, Infante-Amate 2012). No obstante, el notable aumento sobre el interés en el cultivo del almendro (*Prunus dulcis*) en los últimos años posiciona a este cultivo entre los frutales de secano con una mayor superficie destinada en la agricultura española (MAGRAMA, 2018). El olivo, originario del sur del Cáucaso y la zona costera de Siria, se extendió por Chipre hacia Anatolia, y a través de Creta hacia Egipto, hasta poblar todos los países ribereños del Mediterráneo (Besnard *et al.* 2018). Por ello, el olivar es uno de los cultivos leñosos más importantes y tradicionales de la Cuenca Mediterránea; cuyos productos, el aceite de oliva y la aceituna de mesa, son componentes básicos de la dieta tradicional de sus habitantes: la conocida y saludable dieta mediterránea (Rallo 1998, Gorzynik-Debicka *et al.* 2018). En el mundo se cultivan alrededor de 1,500 millones de olivos que ocupan una superficie de más de 10,5 millones de hectáreas, siendo España el primer país olivarero a nivel mundial tanto por la superficie cultivada (2,6 millones de has., 25% de la superficie de olivar mundial) como por la producción (más de 6.600.000 t en la campaña 2016/17, 34,1% de total mundial) (FAOSTAT 2018). Cada año, la superficie destinada a olivar crece en unas 150.000 hectáreas y ya se puede afirmar que cada segundo se plantan diez nuevos olivos en algún lugar del mundo. Hay cerca de 56 países del mundo que producen aceite de oliva y el cultivo del olivar se puede encontrar en lugares antaño inimaginables como China, Australia, Letonia o Finlandia (FAOSTAT 2018). El olivar español está presente en 37 provincias de 15 Comunidades Autónomas, siendo Andalucía donde ocupa la mayor extensión con 1.601.295 has, que suponen el 60,41% del total nacional (MAGRAMA 2018). De hecho, el cultivo del olivo ocupa alrededor del 33% de la superficie agraria útil en la Comunidad Autónoma de Andalucía (INA 2017). Por consiguiente, el olivar andaluz es un elemento emblemático del paisaje de Andalucía y responsable de buena parte de la actividad

económica y social de la Comunidad Autónoma, que confiere a este cultivo un papel singular en la agricultura andaluza (Infante-Amate 2012). Así, las ventas de aceite de oliva y aceituna de mesa representan en torno al 21% de la producción estándar total (PET), generando sobre 2.400 M€ de valor de producción lo que supone alrededor del 3% del PIB de Andalucía, así como establece el 40% del empleo agrícola generado en esta comunidad (INA 2017). Las cifras referidas son claros indicadores de la importancia del sector oleícola en la economía de Andalucía, constituyendo un recurso esencial para una buena parte de la población, tanto por la gran cantidad de mano de obra que ocupa (directa e indirectamente), como por generar utilidades ambientales positivas, relativas a la protección del suelo y la lucha contra la erosión.

Durante los últimos años, en el olivar andaluz se han producido notables innovaciones tecnológicas, que confieren confianza al sector productivo respecto de la capacidad de mantener su estabilidad y competitividad para hacer frente al incremento de exportación de aceite de oliva a nuevos países consumidores. Entre tales innovaciones destacan a) la expansión hacia zonas de regadío, b) el establecimiento de plantaciones con mayor densidad (400 hasta 2.000 árboles/ha) y árboles de un pie adaptados a la recolección mecánica, c) el desarrollo de una industria viverística adaptada a la obtención de plantas de un tronco y entrada precoz en producción y d) la adopción de prácticas de producción, recolección y post-cosecha tendentes a mejorar la calidad del aceite de oliva (Villalobos *et al.* 2006, Rallo *et al.* 2013).

Es evidente que la práctica del riego aumenta considerablemente el rendimiento de la producción en el cultivo del olivo, incluso cuando las aportaciones de agua sean reducidas (Orgaz *et al.* 2017). De hecho, la superficie olivarera en regadío en Andalucía actualmente es de unas 478.000 has, equivalente al 30% del total (MAGRAMA 2018). Asimismo, la amplia distribución del cultivo del olivo en Andalucía hace que esté presente en una amplia gama de gradientes ambientales incluyendo tipos de suelo, variabilidad climática (p.ej. clima subsahariano en Almería), así como en diferentes condiciones orográficas (Ortega *et al.* 2016)

Considerando el material vegetal, la Andalucía olivarera está dividida en cuatro grandes zonas cuya delimitación geográfica y cultivares más

representativos son los siguientes (CAP-JA 2003, Barranco 2017): a) *zona 1* o del Picual (variedad predominante), que comprende la provincia de Jaén y las comarcas de Iznalloz (Granada) y Bujalance (Córdoba); b) *zona 2* o de Hojiblanca (variedad predominante), que incluye la provincia de Córdoba, (excepto las comarcas de Bujalance y La Carlota), y las comarcas de Estepa (Sevilla), Loja (Granada) y Antequera (Málaga); c) *zona 3* o Andalucía occidental, que comprende la provincia de Sevilla (excepto la comarca de Estepa), la comarca de La Carlota (Córdoba) y las provincias de Huelva y de Cádiz, donde los cultivares predominantes son Verdial de Huévar, Lechín de Sevilla, Manzanilla y Gordal Sevillana; y d) *zona 4* o Andalucía oriental, que comprende la provincia de Málaga (excepto la comarca de Antequera), la provincia de Granada (excepto las comarcas de Iznalloz y Loja) y la provincia de Almería, donde los cultivares predominantes son Picual, Hojiblanca, Verdial de Vélez-Málaga, Picual de Almería y Aloreña (CAP-JA 2003, Barranco 2017).

Asimismo, en la actualidad se están implementando métodos alternativos al laboreo convencional para minimizar la erosión, incluyendo: el laboreo ligero, o el no laboreo combinado con control mecánico (desbrozadora), químico (herbicidas) o animal de las malas hierbas, o manteniendo éstas como cubierta vegetal natural o siembra de una cubierta en otoño que es eliminada en primavera mediante la aplicación de herbicidas o mediante pastoreo, entre otros (CAP-JA 2003, Milgroom *et al.* 2007, Rallo *et al.* 2013). Estas cubiertas vegetales proporcionan biomasa que se incorpora al suelo y algunas de ellas se ha demostrado que tienen un efecto supresivo frente a nematodos (Viaene y Abawi 1998). Aunque el modo de acción de estas cubiertas vegetales no se conoce con claridad, pero la liberación de compuestos tóxicos durante el proceso de descomposición se ha sugerido como el posible mecanismo para el control de nematodos. Sin embargo, algunas de estas species también pueden ser huéspedes de NF (p.ej. *Meloidogyne* spp., Liébanas y Castillo 2004).

En resumen, el olivar andaluz está caracterizado por presentar una notable variabilidad en cuanto sistema de manejo del cultivo, genotipos, estar presente en una amplia diversidad de tipos de suelo y zonas climáticas, así como diferentes condiciones orográficas (p.ej. podemos encontrar plantaciones desde nivel del mar hasta 1.400 m.s.m. con

diferentes pendientes, orientaciones, etc.). Todo ello confiere al agroecosistema del **olivar andaluz en un escenario ideal no sólo para el estudio de la biodiversidad de los NF, sino además para conocer cuáles son los factores que realmente estructuran las comunidades de estos organismos en los ecosistemas agrícolas y de este modo, determinar la influencia relativa entre el ambiente y el manejo agronómico en su distribución**.

1.4.2 La relación entre nematodos y el cultivo del olivo

Tal y como se ha indicado en los apartados anteriores, los nematodos constituyen un grupo de organismos residentes en el suelo caracterizado por ser extraordinariamente diverso y complejo, además de estar ampliamente distribuido en todos los sistemas agrícolas. Aunque esta Tesis Doctoral está centrada en las especies que parasitan las plantas, es necesario señalar que la relevancia en el tándem entre agroecosistema y nematodos no sólo subyace en la presencia de especies fitoparásitas. De hecho, la mayoría de los estudios, en un contexto ecológico, que podemos encontrar en la bibliografía están basados en determinar la influencia de la aplicación de diferentes prácticas de manejo agrícola en los ensamblajes de las comunidades de nematodos, compuestas por especies tanto parásitas como de vida libre (es decir, aquellas no parásitas) (Sánchez-Moreno *et al.* 2009, Culman *et al.* 2010, Zhang *et al.* 2017). En el caso particular del olivar andaluz, estudios recientes certifican el uso de las comunidades de nematodos como potentes indicadores de la calidad del suelo, estableciendo además estrechas relaciones con la aplicación de diferentes prácticas agrícolas y/o el uso de diferentes variedades de este cultivo (Palomares-Rius *et al.* 2012, Sánchez-Moreno *et al.* 2015). Resaltado este aspecto, a continuación, se desarrollará un estado del arte sobre los NF en el cultivo del olivo, resaltando aspectos relacionados con el impacto en la producción, patogenicidad y la biodiversidad.

Prácticamente cualquier cultivo agrícola, incluido el olivo, puede sufrir un perjuicio importante debido al parasitismo de alguna especie de nematodo fitoparásito. La magnitud de las pérdidas que ocasionan depende fundamentalmente de la densidad de población en suelo y/o

raíces, de la susceptibilidad del cultivo, y de las condiciones ambientales (Castillo *et al.* 2010). Tal y como se indicó en el epígrafe, el complejo de enfermedades que causa el parasitismo de NF en el olivar no suele ser considerado como un problema fitosanitario de relevancia. A esto hay que añadir que el daño producido por NF en las plantaciones de olivar ya establecidas puede ser no relacionado claramente por la presencia de estos organismos debido a la no especificidad en la sintomatología en la parte aérea de la planta (Castillo *et al.* 2010). No obstante, estudios realizados en Estados Unidos estimaron entre 5-10% las pérdidas en la producción en el cultivo del olivo ocasionadas por el parasitismo de *Tylenchulus semipenetrans* y especies del género *Meloidogyne* (Koenning *et al.* 1999). En los últimos años se ha descrito por diversos autores que el efecto de los NF en el cultivo del olivo produce mayores pérdidas de vigor y decaimiento en los estadios de desarrollo iniciales de la planta previas a su plantación (es decir, plantas de vivero) (Nico 2002, Sasanelli 2009, Castillo *et al.* 2010). Por ejemplo, *Meloidogyne javanica* fue uno de los principales patógenos asociados con la alta incidencia del "síndrome de la seca" en los olivares recién establecidos en Argentina (Pérez *et al.* 2001). La relevancia de este aspecto se ve incrementada en el actual modelo que caracteriza la olivicultura moderna debido a que se basa en el establecimiento de nuevas plantaciones o al reemplazamiento de las establecidas por plantas más jóvenes (Rallo *et al.* 2013). Todo ello ha sido favorecido por el desarrollo de la industria viverística en Andalucía a partir de la puesta a punto y difusión del método de propagación de olivo por enraizamiento de estaquillas semileñosas bajo nebulización incrementando la calidad de las plantas obtenidas (Caballero 1980), lo que ha contribuido notablemente a la expansión y modernización del olivar en los últimos años.

Considerando lo expuesto en el párrafo anterior unido a la complejidad intrínseca del ecosistema del suelo, es evidente que el método más apropiado para luchar contra las infecciones provocadas por nematodos es evitar el contacto directo entre planta y parásito (Nico 2002). De este modo la utilización de plantones libres de inóculo (es decir, especímenes de NF), así como la elección de suelos no infestados o con baja densidad de inóculo, son las estrategias más recomendables para el establecimiento de olivares fitopatológicamente sanos. Todo ello ha llevado a la necesidad de

establecer la certificación sanitaria del material vegetal de plantas de vivero. Mediante la redacción del Real Decreto 929/1995 del Ministerio de Agricultura, Pesca y Alimentación (BOE-A-1995-14422 de 14/06/1995) y posteriormente con la modificación del mismo con el Real Decreto 744/2016 (31 de diciembre de 2016), se establecieron las pautas y normas para la certificación del material vegetal mediante su legislación. Por otro lado, dicho aspecto también ha sido reconocido a nivel autonómico mediante la legislación reciente a través del Reglamento Específico de Producción Integrada del Olivar de la Junta de Andalucía (BOJA nº 117 de 16/06/2010, págs. 6-18). En este contexto, la necesidad de utilización de material certificado libre de patógenos, incluidas diferentes especies de NF, es destacada para la puesta en práctica de la Producción Integrada de olivar en Andalucía (Pérez Mohedano y Ortíz Berrocal 2011). De hecho, en prospecciones fitopatológicas realizadas en viveros de olivo en las provincias andaluzas con importante tradición olivarera (Córdoba, Jaén y Sevilla) revelaron infecciones por los nematodos noduladores de raíz (*Meloidogyne arenaria*, *M. incognita* y *M. javanica*), y los nematodos lesionadores de raíz (*P. penetrans* y *P. vulnus*), demostrando que los plantones de olivo infectados por NF constituyen un medio potencial para la dispersión de estos agentes a nuevas áreas olivareras (Nico *et al.* 2002). Además, estas especies fueron patogénicas sobre los dos cultivares de olivo evaluados provocando una reducción del crecimiento manifestada fundamentalmente en el diámetro caulinar en los cvs. Picual y Arbequina (Nico *et al.* 2003). Es necesario destacar que aunque las cuatro especies más cosmopolitas del género *Meloidogyne* (*arenaria*, *hapla*, *incognita*, *javanica*) parasitan y se reproducen sobre plantas leñosas en todo el mundo, incluyendo la mayoría de las especies de frutales, *M. javanica* es la especie que ha demostrado una mayor incidencia y severidad en dichos huéspedes (Abrantes *et al.* 1992, Téliz *et al.* 2007, Sasanelli 2009, Castillo *et al.* 2010).

Otro aspecto a destacar y que no se puede obviar es la potencial interacción sinérgica entre NF y el hongo fitopatógeno del suelo *V. dahliae* (Castillo *et al.* 2010). No obstante, el incremento notable en la extensión y severidad de la Verticilosis del olivo, unido a la amplia distribución que caracteriza a los NF en cualquier ecosistema agrícola (Neher 2010), hace plausible que ambos patógenos puedan coexistir en una misma área e

infectar a la planta. Además, existen evidencias experimentales sobre la existencia de una interacción sinérgica entre nematodos fitopatógenos y *V. dahliae* en diversos cultivos (Mckinley y Talboys 1979, Riedel *et al.* 1985, Rowe *et al.* 1985, Wheeler y Riedel 1994) incluyendo olivo (Lamberti *et al.* 2002, Saeedizadeh *et al.* 2003). Estos datos demuestran que la coinfección de material de plantación por estos nematodos, tiene importantes consecuencias desde el punto de vista fitopatológico y puede constituir un riesgo para el establecimiento de nuevas plantaciones de olivo. Además, la amplia distribución que tanto las especies de nematodos descritas como *V. dahliae* tienen en las diversas áreas olivareras hace plausible que ambos patógenos estén coexistiendo en un mismo suelo y puedan co-infectar la planta.

Por otro lado, al igual que en otras plantas huéspedes, los ataques por nematodos formadores de nódulos (*Meloidogyne* spp.) y por los lesionadores de raíz (*Pratylenchus* spp.) reducen el crecimiento y vigor del olivo en todos los países donde se han descrito infecciones por alguna de estas especies (Lamberti y Baines 1969a 1969b, Abrantes *et al.* 1992, McKenry 1994). Además, el primer grupo de estos nematodos origina una serie de nodulaciones en la raíz como consecuencia de su actividad parasítica, que reduce la capacidad de absorción de agua y nutrientes por la misma (Sasanelli 2009, Castillo *et al.* 2010). No obstante, es necesario señalar que la reacción a la infección de dos especies de nematodos noduladores (*Meloidogyne incognita* y *Meloidogyne javanica*) (Lamberti y Baines 1969b) puede ser diferencial dependiendo del cultivar de olivo y de la especie de nematodo fitopatógeno (Sasanelli *et al.* 1997, Castillo *et al.* 2010). Generalmente la resistencia de la planta huésped probablemente sea la estrategia más eficiente para minimizar los efectos del parasitismo por NF (y otros patógenos). Aunque el uso de portainjertos de olivos resistentes a NF (p.ej. 'Allegra') ha sido demostrado como una buena estrategia de manejo en experimentos realizados en California, Estados Unidos (McKenry 1994), hasta el momento, el esfuerzo en I+D+i en este sentido ha sido limitado en el cultivo del olivo.

El grado de resistencia y susceptibilidad del olivo frente a NF puede variar considerablemente dependiendo del cultivar utilizado (Sasanelli *et al.* 1997, Castillo *et al.* 2010). Por ejemplo, en un estudio llevado a cabo en

Egipto se evaluó la reacción de seis cultivares de olivo frente al nematodo nodulador *Meloidogyne incognita* y al nematodo reniforme *Rotylenchulus reniformis* (Al-Sayed y Abdel-Hameed 1991). En este estudio los cultivares Meashon y Tofahy fueron identificados como resistentes a aislados de *M. incognita* y *R. reniformis* de Egipto, mientras que los cultivares Manzanilla y Egazi fueron moderadamente susceptibles a *M. incognita* pero tolerantes a *R. reniformis* entre otras divergencias encontradas (Al-Sayed y Abdel-Hameed 1991). Similarmente en un estudio realizado en Italia se evaluaron los cultivares de olivo más comúnmente utilizados frente a *M. incognita* y *M. javanica*; observándose que el cultivar Coratina fue resistente a ambos, 'Leccino' y 'Yusti' fueron resistentes a *M. javanica* y moderadamente susceptibles a *M. incognita*; mientras que otros cultivares como Cima di Bitonto, Cellina di Nardo, Frantoio y FS 17 fueron moderadamente susceptibles a *M. incognita* (Sasanelli *et al.* 1997, 2002). Sin embargo en España, no se ha evaluado la reacción de los cultivares de olivo frente a nematodos fitopatógenos (particularmente *Meloidogyne* spp. y *Pratylenchus* spp.), y los únicos datos disponibles son la respuesta de 'Picual' y 'Arbequina' (Nico *et al.* 2002, 2003, Castillo *et al.* 2010). Por este motivo, sería de gran utilidad disponer de datos contrastados sobre la respuesta de los cultivares de olivo de mayor interés y extensión frente a infecciones por aislados españoles de los nematodos noduladores y lesionadores de raíces prevalentes en Andalucía.

La biodiversidad de NF asociada al cultivo del olivo ha aumentado considerablemente en los últimos años. Como ya se ha indicado en diversas ocasiones el cultivo del olivo es el cultivo leñoso por excelencia de la Cuenca Mediterránea (Besnard *et al.* 2018). Sin embargo, aspectos tales como la extensa distribución de esta planta (ya sea en su forma cultivada como silvestre, *Olea europea* var. *sylvestris*) como el hecho de la escasa especialización que se atribuye a los principales nematodos asociados a plantas leñosas en climas templados, son atributos que subyacen de este notable aumento. A esto hay que añadir la aparición de ciertos países olivareros emergentes, como Argentina, Chile y Perú entre otros (Nico 2002, Besnard *et al.* 2018), sin olvidar además la influencia del extraordinario avance en la descripción de taxones ocurrido en los últimos años (Seesao *et al.* 2017). La primera referencia de especies de NF asociadas a la rizosfera del cultivo del olivo fue citada en Estados Unidos,

donde nematodos noduladores del género *Meloidogyne* fueron detectados (Buhrer *et al.* 1933). En una revisión bibliográfica sobre las citas de NF asociados a raíces y/o rizosfera del olivo ascendió hasta las 70 especies diferentes pertenecientes a 33 géneros (Lamberti y Vovlas 1993). En los siguientes años, en prospecciones ocasionales se procedió a la descripción de nuevos taxones así como nuevas citas de especies asociadas a la rizosfera del olivo (Nico *et al.* 2002, Vovlas *et al.* 2002, Sasanelli 2009). Por ejemplo, una nueva especie del género *Meloidogyne* (*M. baetica*) fue encontrada parasitando la rizosfera de olivar silvestre (Castillo *et al.* 2003b); o se detectaron por primera vez el nematodo formador de quistes *Heterodera mediterranea* en suelos arenosos de Sevilla (Castillo *et al.* 1999), y *Rotylenchulus macrosoma* en olivos silvestres (Castillo *et al.* 2003c). Además, a partir de un estudio realizado en un banco de germoplasma de olivo reveló un efecto significativo del genotipo del olivo sobre comunidades de NF (Palomares-Rius *et al.* 2012). Toda esta información generada creó la necesidad de actualizar y discutir la biodiversidad de nematodos asociados al olivo (Ali *et al.* 2014).

La revisión bibliográfica realizada por Ali *et al.* (2014) reveló una extraordinaria capacidad por parte de la planta del olivo de hospedar a una sorprendente diversidad de especies de NF. Este análisis de la literatura encontró un total de 153 especies diferentes pertenecientes a 56 géneros distribuidos en los siguientes órdenes del filo Nematoda: Aphelenchina (4 géneros y 3 especies), Dorylaimida (5 géneros y 36 especies) y Tylenchina (48 géneros y 114 especies) (Ali *et al.* 2014). Cabe destacar que la mayoría de los nematodos citados como parásitos del olivo han sido también detectados en otros cultivos y plantas silvestres (Sikora *et al.* 2018). Considerando la riqueza de especies encontradas, los géneros con una mayor diversificación de especies asociadas al olivo fueron *Xiphinema*, *Tylenchorhynchus*, *Pratylenchus*, *Helicotylenchus*, *Longidorus*, *Meloidogyne* y *Paratylenchus* con un rango de entre 19 a 8 especies, respectivamente; mientras que aproximadamente el 42 % de los géneros fueron representados con sólo una especie (Ali *et al.* 2014). Desde una perspectiva también a nivel mundial, sin embargo, los géneros con un mayor impacto económico fueron ordenados descendientemente de la siguiente manera: *Meloidogyne*, *Pratylenchus*, *Helicotylenchus*, *Xiphinema*, *Tylenchulus*, *Rotylenchulus* y *Heterodera* (Castillo *et al.* 2010, Ali *et al.*

2014). Asimismo, la distribución de la mayoría de especies de NF es extensa y amplia en todas las zonas donde el olivo está presente. Países con tradición olivarera como España, Marruecos, Jordania, Italia o Grecia son los países con una mayor diversidad de géneros de nematodos parasitando la rizosfera de la planta de olivo (Lamberti y Vovlas 1993, Vovlas *et al.* 2002, Sasanelli 2009, Castillo *et al.* 2010, Ali *et al.* 2014, 2017). Sin embargo, esta tendencia puede estar supeditada a un componente de variabilidad dada la potencial influencia de la presencia científica de nematólogos con vocación taxonómica y agronómica presente en la zona olivarera analizada.

En los últimos años son varios los estudios que han revelado nuevos conocimientos sobre la distribución de especies o nuevas citas de nematodos parasitando la rizosfera de plantas de olivo, así como la descripción de nuevas especies (Ali *et al.* 2015, De Luca *et al.* 2014, Guesmi-Mzoughi *et al.* 2016, Tzortzakakis *et al.* 2014, 2015, 2016b, 2018 Archidona-Yuste *et al.* 2018, Palomares-Rius *et al.* 2018a, 2018b). De todos estos estudios podemos destacar la descripción de dos nuevas especies del género *Meloidogyne* infectando raíces de olivar silvestre y cultivado en España, *M. oleae*, y olivo silvestre en Marruecos, *M. spartelensis* (Ali *et al.* 2015, Archidona-Yuste *et al.* 2018). La descripción de dos nuevas especies del género *Xiphinema* (*X. cretense* y *X. herakliense*) encontradas parasitando en la rizosfera de olivar silvestre y cultivado en la isla de Creta, Grecia (Tzortzakakis *et al.* 2014, 2015). Además de un estudio enfocado a determinar la prevalencia de nematodos semiendoparásitos del género *Rotylenchulus* en olivo en la Cuenca Mediterránea, estableciendo la primera de cita de la especie *R. macrosoma* infectando raíces de olivo cultivado en Grecia (Palomares-Rius *et al.* 2018a). Por otro lado, se han realizado otros estudios con el objetivo finalista de determinar los potenciales factores que estructuran la distribución de importantes grupos de NF como el género *Meloidogyne* en Andalucía y Marruecos (Aït Hamza *et al.* 2017, Archidona-Yuste *et al.* 2018); revelando efectos significativos de propiedades edáficas y prácticas de manejo agronómico como factores potenciales que estructuran su distribución. Finalmente, las mismas zonas olivareras han sido el foco de estudios con base ecológica con el objetivo de incrementar el conocimiento sobre los factores que determinan la distribución espacial de comunidades

de NF del olivo cultivado (Palomares-Rius *et al.* 2015, Ali *et al.* 2017). En el estudio llevado a cabo en Andalucía se observó una estrecha relación entre las propiedades del suelo y genotipo del olivo con las comunidades de nematodos (Palomares-Rius *et al.* 2015); y factores de índole antropogénico (p.ej. la domesticación del olivo frente su forma silvestre) como los factores con una mayor importancia en la distribución de estas especies en Marruecos (Ali *et al.* 2017). Este último estudio reveló además una extraordinaria diversidad de especies asociadas al olivo no conocida hasta la fecha, incrementando además la biodiversidad mundial de NF asociadas a esta planta hasta 223 especies documentadas (Lamberti y Vovlas 1993, Sasanelli 2009, Castillo *et al.* 2010, Ali *et al.* 2014, 2017).

Todo lo expuesto anteriormente evidencia la importancia y necesidad de realizar estudios de biodiversidad mediante un enfoque sistemático en la obtención de resultados fiables sobre los factores que influyen en la distribución de comunidades de los organismos en estudio. Ejemplos de ello, podemos encontrarlos para una amplia gama de organismos de impacto económico en un contexto agrícola así como en estudios destinados a la protección de organismos como método de conservación de ecosistemas en peligro de degradación (Ettema y Wardle 2002). Su aplicación en los NF en un contexto agrícola es esencial para conocer y adoptar los mejores métodos de manejo de las comunidades de estos organismos. Podemos encontrar ejemplos en estudios realizados recientemente en la zona olivarera de Marruecos (Ali *et al.* 2017). En cambio, en España en general y en Andalucía en particular, las prospecciones sistematizadas de NF en olivares comerciales han sido casi inexistentes, destacando la realizada en olivares adulos en la provincia de Jaén (Peña-Santiago 1990) indicando infestaciones por varios ectoparásitos migratorios (*Helicotylenchus* spp., *Xiphinema* spp., *Longidorus* spp., *Trichodorus* spp.); o la más reciente llevada a cabo en 89 parcelas comerciales distribuidas por el olivar Andaluz (Palomares-Rius *et al.* 2015), demostrando la existencia de altas infestaciones por más de 70 especies distintas, algunas de ellas con elevadas densidades de población en campos de olivar de Andalucía. Sin embargo, algunas de las condiciones agronómicas derivadas del desarrollo de las nuevas técnicas de cultivo en los últimos años, así como la consideración sistemática de

todos los sistemas de producción y áreas en el olivar andaluz no han sido contempladas hasta la fecha.

1.5 Estructura y objetivos de la tesis

La generación de resultados útiles y fiables para comprender el estado actual y predecir la incidencia de patógenos y parásitos transmitidos por el suelo en un escenario futuro, debe de realizarse a través de un enfoque integrativo basado en el estudio de los factores ambientales que potencialmente determinen la distribución espacial y ciclos de vida de los organismos en estudio incluidos en el ecosistema del suelo. De hecho, la implantación de este enfoque puede proporcionar eficaces directrices para el manejo de enfermedades de suelo actuales y emergentes (Wall *et al.* 2015). Por tanto, es evidente que la aplicación de dicho planteamiento es ideal para el escenario actual del olivar en Andalucía unido a la potencial amenaza económica que subyace de la presencia de una alta diversidad de NF.

Tradicionalmente, el olivar andaluz ha sido considerado como un sistema agrícola estable caracterizado por escasa alteración antrópica y una marcada adaptación del material vegetal a las condiciones ambientales. Además estas condiciones han establecido un equilibrio sutil entre el olivo y sus patógenos, produciéndose pérdidas considerables de sanidad vegetal cuando se rompe ese equilibrio (Albajes *et al.* 2018). Sin embargo, de manera general y en particular en Andalucía, la olivicultura está sometida a cambios rápidos y difíciles de predecir que obligan a replantear estrategias de producción mediante innovaciones tecnológicas, el empleo de plantones obtenidos por enraizamiento de estaquillas semileñosas que pueden constituirse en vehículo de formas infectivas de muchas especies de NF (Nico 2002, Castillo *et al.* 2010), así como la súbita expansión del olivar a nuevas áreas con características edáficas y climáticas diferentes a las propias del olivar tradicional. Todas estas alteraciones agronómicas y condiciones ambientales pueden resultar muy favorables para la reproducción de algunas especies de NF de marcada

importancia económica y potencial en el olivo caracterizándose a su vez como enfermedades emergentes (Perry y Moens 2006, Castillo *et al.* 2010). Por estas razones, es previsible deducir que el marco formado por los NF y el olivar en Andalucía se postula como un escenario ideal para el estudio de la biodiversidad y ecología para el desarrollo de medidas para su manejo y control. Además, el hecho de que el agroecosistema del olivar andaluz muestre una amplia gama de sistemas de producción, de manejo y genotipos de olivo así como su presencia bajo amplios gradientes ambientales incluyendo clima, suelo y/o variabilidad orográfica (Rodrigo *et al.* 2012, Ortega *et al.* 2016, RAIF 2016, REDIAM 2016), lo hacen más idóneo para aumentar el conocimiento sobre las directrices que determinan la distribución espacial de estos organismos en un contexto amplio en la ecología de los agroecosistemas.

Como ya ha sido resaltado en diversas ocasiones, la información sistematizada sobre la distribución de NF que infectan el olivar en Andalucía actualmente es incompleta. La divergencia en la biodiversidad asociada al olivo entre los estudios realizados en Andalucía detectando 70 especies diferentes (Palomares-Rius *et al.* 2015), y el llevado a cabo en Marruecos donde se identificaron un total de 117 especies de NF (Ali *et al.* 2017), podría ser un síntoma de dicha falta de información. Además, recientemente prospecciones sistemáticas en las zonas de cultivo de viñedo en Andalucía revelaron una extraordinaria diversidad de NF ya que se identificaron un total de 173 especies diferentes (Navas-Cortés y Castillo 2014). La mayoría de las especies encontradas en este estudio se caracterizan por tener la capacidad de parasitar una amplia gama de plantas huésped (Perry y Moens 2006, Sikora *et al.* 2018). Como ya se ha indicado, el cultivo de la vid en Andalucía se asemeja en importancia y en distribución (aunque en menor grado) al cultivo del olivo (Rallo 1998). A partir de las similitudes entre ambos cultivos y los nematodos que parasitan sus raíces, resulta necesario evaluar si la biodiversidad de estos organismos es análoga o diferente en el cultivo del olivo. Por otro lado, los datos de diversidad detectados en el cultivo de la vid evidenciaron una extraordinaria presencia de especies pertenecientes a la familia Longidoridae (32 especies pertenecientes a los géneros *Xiphinema* y *Longidorus*), siendo esta familia la que presentó el mayor número de especies detectadas (Gutiérrez-Gutiérrez 2011). La capacidad de las

especies de la familia Longidoridae como como vectores de virus vegetales enfatiza la relevancia fitopatológica en el estudio de la prevalencia y distribución de este grupo de nematodos (Nicol *et al.* 2011). Además estos nematodos son considerados como los principales patógenos en importantes zonas olivareras del mundo (p.ej. Chile y Estados Unidos), donde se han estimado unas pérdidas que oscilan entre el 5 al 10% de la producción (Ali *et al.* 2014). Todo ello suscita el interés y justifica el estudio sistematizado de la diversidad y prevalencia de los nematodos longidóridos infestando suelos del cultivo de olivo en Andalucía, aspecto que no ha sido investigado hasta la fecha.

Por otro lado, son diversos los estudios que han revelado efectos significativos de gradientes ambientales y agronómicos (p.ej. propiedades del suelo, factores climáticos, sistema de manejo, etc.) en la distribución de NF en diversos sistemas agrícolas incluidos el olivar (Duyck *et al.* 2012, Palomares-Rius *et al.* 2015, Sánchez-Moreno *et al.* 2015, Aït Hamza *et al.* 2017, Ali *et al.* 2017). No obstante, la fiabilidad de los resultados obtenidos está estrechamente relacionada con la escala considerada en cada estudio, dado su papel intrínseco en la compartimentación de la variación de los fenómenos ecológicos. Es decir, la escala-dependencia de la heterogeneidad espacial de los factores ambientales es clave a la hora de encontrar relaciones significativas entre procesos ecológicos y patrones de biodiversidad (Wiegand *et al.* 2017). En este sentido, numerosos estudios han demostrado estructuras espaciales en la distribución de comunidades de plantas y animales, pero se conoce muy poco sobre la estructura de la diversidad de organismos del suelo (Soininen 2016), siendo este aspecto rara vez considerado en los estudios sobre comunidades de NF. De hecho, hay preguntas clave que aún no han sido consideradas en este tipo de estudios, tales como: **¿cuál es el papel de los patrones espaciales en la variación de las comunidades de NF?**, o **¿la influencia de los factores ambientales está espacialmente estructurada?** La respuesta a estas cuestiones queda suscitada en la incorporación de la fluctuación espacial en los modelos ecológicos a partir de un enfoque estadístico que permita caracterizar las relaciones espaciales del diseño del muestro como covariables (Dray *et al.* 2006), metodología ampliamente contrastada en una amplia gama de ecosistemas (Legendre y Legendre 2012).

La mayoría de los estudios ecológicos basados en la distribución espacial de las comunidades de NF han sido desarrollados utilizando la diversidad alfa o gamma como variables descriptivas de la biodiversidad biológica (Recuadro 1). Sin embargo, en comparación con la diversidad beta, estos enfoques no permiten probar hipótesis sobre los procesos que subyacen a la distribución de las especies y la biodiversidad ya que ignoran la identidad de las especies (Tuomisto 2010). En definitiva, la aplicación de la diversidad beta como medida de la biodiversidad proporciona un conocimiento cuantitativo que vincula los procesos locales y regionales que impulsa los patrones de diversidad de especies (Anderson *et al.* 2011). Además, permite estimar mediante el cálculo de índices la singularidad ecológica de las unidades de muestreo y de cada especie en función de su contribución en la variación global de la comunidades de especies entre sitios, es decir la diversidad beta (Legendre y De Cáceres 2013). Por consiguiente, la variación de este índice puede corresponder a varios aspectos relacionados con la composición excepcional de especies (p.ej. presencia de especies poco frecuentes), sitios de alta conservación o degradación (p.ej. efecto de aplicación productos fitosanitarios), condiciones ecológicas particulares (p.ej. efecto de sistemas de manejo) o el efecto de especies invasoras en las comunidades, entre otros ejemplos (Legendre y Gauthier 2014). Son numerosas las ventajas que subyacen en la aplicación de la diversidad beta sobre el análisis de patrones de biodiversidad, siendo necesario cuando el objetivo es determinar los factores ambientales que estructuran la distribución espacial de comunidades (Tuomisto 2010, Anderson *et al.* 2011, Legendre y De Cáceres 2013). Sin embargo, los estudios sobre la diversidad de especies de NF basados en la diversidad beta son inexistentes, ya que sólo podemos encontrar un estudio reciente donde su aplicación fue incompleta (Palomares-Rius *et al.* 2015).

Las circunstancias expuestas anteriormente motivan a establecer como **objetivo finalista** de la Tesis Doctoral **el estudio de la diversidad de los NF en el olivar de Andalucía y determinar los procesos ecológicos que determinan su distribución y la variación espacial de las comunidades**. Dada la amplia diversidad de sistemas producción y gradientes ambientales que se atribuyen al olivar en Andalucía, esta Tesis Doctoral revelará nuevas ideas para mejorar las prácticas de manejo y

anticipar respuestas a la posibilidad potencial de la presencia de enfermedades emergentes ante los cambios tecnológicos que está imponiendo la nueva olivicultura, así como a los cambios climáticos que pueden afectar a la severidad de estos organismos. El **carácter innovador** de esta Tesis Doctoral incluye varios aspectos tales como: el enfoque sistemático en el diseño del muestro abordando todas características ambientales y agronómicas del olivar andaluz lo que permitirá obtener datos fiables sobre la biodiversidad de NF asociada a este cultivo en Andalucía; la evaluación de la prevalencia, diversidad y distribución de los nematodos longidóridos asociada a este cultivo; y por último, determinar la influencia de los factores ambientales y agronómicos que determinan los patrones espaciales de las comunidades de nematodos mediante la incorporación de la fluctuación espacial de las comunidades y el uso de índices de diversidad basados en la diversidad alfa/gamma y beta. Dada la complejidad y magnitud del enfoque científico planteado, la presente tesis está dividida en tres grandes bloques, dedicado cada uno de ellos a los tres objetivos específicos siguientes:

1) Determinar la **biodiversidad de nematodos fitoparásitos** asociada a la rizosfera de olivares en Andalucía incluyendo aspectos tales como la identidad, prevalencia, densidad de población y distribución geográfica de cada una de las especies detectadas.

2) Diagnóstico, prevalencia y distribución de nematodos fitoparásitos pertenecientes a los géneros ***Xiphinema*** **y** ***Longidorus*** en el cultivo del olivo en Andalucía.

3) Determinar la influencia de los **factores ambientales**, incluyendo propiedades edáficas y climáticas**, y agronómicos** en la **distribución espacial de las comunidades** de nematodos asociadas al cultivo del olivo en Andalucía

A modo de resumen, los distintos capítulos que se incluyen en cada bloque se presentan a continuación.

Bloque I. Nematodos fitoparásitos asociados al olivar en Andalucía.

Este bloque está compuesto por el **capítulo 1** (**Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in southern Spain**), en el que se establece el diseño del muestreo sistemático, utilizando un total de 376 parcelas ampliamente distribuidas por toda la superficie del cultivo del olivo en Andalucía teniendo en cuenta todos los sistemas de producción contemplados en la olivicultura actual. Además en este capítulo se detallan los métodos utilizados para la extracción de los nematodos y su posterior identificación morfológica y molecular (Castillo *et al.* 2003b), así como la amplia gama de variables asociadas a cada punto de muestreo para determinar la influencia de los factores ambientales (clima, suelo y topografía) y agronómicos en los patrones espaciales de las comunidades de nematodos detectadas. En particular, el diagnóstico de especies de nematodos noduladores de raíces (*Meloidogyne* spp.) se realizará mediante un protocolo estandarizado que incluye un enfoque multidisciplinar (Castillo *et al.* 2003b). Brevemente, además de la identificación integrativa de los juveniles encontrados en las muestras del suelo, las raíces noduladas se diseccionarán para el posterior aislamiento de hembras adultas enteras con objeto de identificar la especie mediante estudios polifásicos, incluyendo estructura del patrón perineal y posición del poro excretor (Jepson 1987), análisis bioquímico de isoenzimas, esterasas y malatodeshidrogenasas (Esbenshade y Triantaphyllou 1985), y análisis molecular de ADN ribosómico (ITS, 28S, 18S) y cebadores específicos diseñados a partir de fragmentos SCARs ("Sequence Characterized Amplified Region") (Castillo *et al*. 2003b, Gutiérrez-Gutiérrez *et al*. 2011). Además, las poblaciones de huevos y juveniles de nematodos noduladores en raíces se evaluarán mediante el método de extracción en 0.5% hipoclorito de sodio (Hussey y Barker 1973). Finalmente, también se muestran los resultados sobre la biodiversidad de NF encontrada parasitando la rizosfera del olivo en Andalucía. Los resultandos han sido publicados en la revista Data in Brief.

Bloque II. La familia Longidoridae en el olivo de Andalucía.

En el bloque II se estudia la prevalencia y distribución de NF pertenecientes a los géneros *Xiphinema* y *Longidorus* en olivo en Andalucía. Antes de describir brevemente la metología llevada a cabo en este bloque, es necesario mencionar que además del olivo en su forma cultivada el diagnóstico de este grupo de nematodos también será realizado para el olivar silvestre con el fin de realizar una comparación entre ambiente natural y agrícola al tal respecto. Al igual que en otros grupos de nematodos, la identificación de este grupo es altamente compleja debido al gran número de especies identificadas, la presencia de especies crípticas y al solapamiento entre caracteres entre especies. Por ello, el diagnostico en este grupo debe de hacerse mediante un enfoque multidisciplinar integrando técnicas moleculares, de microscopia y herramientas de análisis multivariante (Gutiérrez-Gutiérrez 2011). La identificación de estos nematodos mediante el uso de marcadores moleculares altamente conservados de ADNr nuclear (región D2-D3 del gen 28S, 18S, región ITS1) y ADNmt ha mostrado excelentes resultados (Ye *et al.* 2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2013, Peneva *et al.* 2013, Peraza-Padilla *et al.* 2016). No obstante, el empleo de dichos marcadores proporciona una herramienta adicional pero no una alternativa independiente al análisis exhaustivo de la morfología. En este sentido, se evaluará el uso de las herramientas estadísticas de análisis multivariante en la identificación de especies crípticas dado los buenos resultados obtenidos en otros grupos de nematodos (Cantalapiedra-Navarrete *et al.* 2013). La presencia de dos grupos (americanum y no americanum) en el género *Xiphinema* (Loof y Luc 1990, Lamberti *et al.* 2000) ha sido considerada a la hora de establecer los capítulos incluidos en este bloque. En el **capítulo 2** (**Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematoda: Longidoridae) as inferred from morphometrics and molecular markers**) se describe la diversidad de especies del grupo americanum de *Xiphinema* detectadas en olivo describiendo tres especies nuevas y se evalúa el uso integrado de métodos moleculares, morfométricos y análisis multivariante en la identificación de especies de este grupo. El **capítulo 3** (**Molecular phylogenetic analysis and comparative morphology resolve two new species of olive-tree soil related dagger nematodes of the genus**

Xiphinema **(Dorylaimida: Longidoridae) from Spain**) se incluye la descripción de dos nuevas especies pertenecientes al grupo no americanum de *Xiphinema* detectadas en la rizosfera de olivo. En el **capítulo 4** (**Remarkable diversity and prevalence of dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches**) se revela la diversidad, prevalencia y distribución de nematodos del género *Xiphinema* en el olivo en Andalucía con la descripción de cuatro especies nuevas, evaluando además la variabilidad entre el olivo cultivado y silvestre. Finalmente, el **capítulo 5** (**Unravelling the biodiversity and molecular phylogeny of needle neamatodes of the genus *Longidorus* (Nematoda: Longidoridae) in Olive and a description of six new species**) discute, en este caso, la diversidad, prevalencia y distribución de nematodos del género *Longidorus* en el olivo en Andalucía con la descripción de seis especies, comparando los datos obtenidos en el olivo cultivado y silvestre. Los resultandos han sido publicados en las revistas PloS One, Zoological Journal of Linnean Society e Invertebrate Systematics.

Bloque III. Descifrando la diversidad beta de nematodo fitoparásitos asociados al cultivo del olivo.

En este último bloque se evalúa la influencia de factores ambientales y agronómicos en la distribución espacial de las comunidades de NF en el olivo cultivado en Andalucía. En el **capítulo 6** (**Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olives in southern Spain**) se ha evaluado la influencia de variables ambientales (suelo, clima, incluyendo topografía, y manejo agronómico) y de la estructura espacial que determinan la riqueza y diversidad beta de NF que infestan suelos de olivar en Andalucía. Se han utilizado técnicas de partición de la varianza para evaluar las contribuciones relativas y compartidas entre los diferentes bloques de variables. Además, con el fin de identificar áreas y especies de particular interés, la diversidad beta se dividió en dos índices relacionados con la contribución de los puntos de muestreo (LCBD) y las especies (SCBD) en la variación de comunidades de NF. Finalmente se discute la influencia de cada uno de los tipos de variables (suelo, clima y manejo agronómico) en

la distribución espacial y variación entre las comunidades de NF detectadas, así como el análisis de la estructura espacial y la influencia de la estocasticidad. Los resultados han sido publicados en la revista Agriculture, Ecosystemas and Environment.

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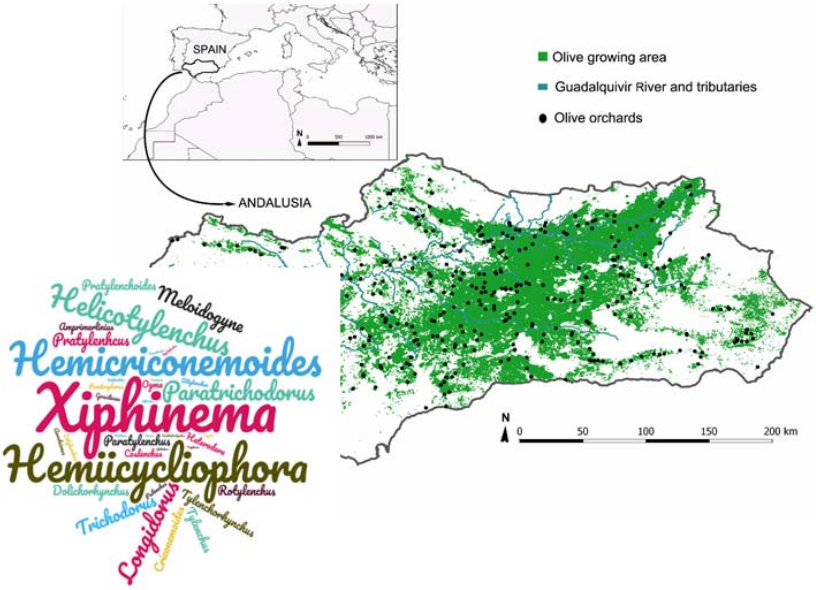
2 BLOQUE I

NEMATODOS FITOPARÁSITOS ASOCIADOS AL OLIVAR EN ANDALUCÍA



C1

Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in southern Spain

Antonio Archidona-Yuste, Thorsten Wiegand, Pablo Castillo, Juan A. Navas-Cortés

***Data in Brief*, enviado**

Abstract

In this data article, we aimed to unravel the diversity of plant-parasitic nematodes (PPN) associated with cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) in southern Spain, Andalusia. The olive growing area of Andalusia is of high agriculture and socio-economic importance with an extensive distribution of this crop. To this end, we conducted a systematic survey comprising 376 commercial olive orchards covering the diversity of cropping systems applied. Data showed 128 species of PPN belonging to 38 genera and to 13 families. In addition, an extensive data set regarding to potential factors in structuring the community patterns of PPN found in the 376 commercial olive orchards sampled is provided. Three variables data set were compiled including above-ground environment, soil and agronomic management. Overall, 48 explanatory variables were selected as determinist processes on shaping the diversity of PPN. Finally, data also showed the values regarding to the partition of beta diversity into contributions of single sites to overall beta diversity (LCBD) and intro contributions of individual species to overall beta diversity (SCBD). Data may serve as benchmarks for other groups working in the field of PPN diversity associated with crops and of below-ground communities and ecosystems.

ADDITIONAL KEYWORDS: nematode diversity – plant-parasitic nematodes – cultivated olive

Specifications Table

Subject area	*Ecology*
More specific subject area	*Plant-parasitic nematode ecology. A case of study: cultivated olive in southern Spain*
Type of data	*Tables and figures*
How data was acquired	*Nematode identification was acquired by using integrative taxonomy (using a Zeiss III compound microscope with Nomarski differential interference contrast at up to ×1000 magnification and molecular methods* standardized*). Variable data sets were compiled from GIS, directly provided by landowner and/or data collection*
Data format	*Raw and analyzed*
Experimental factors	*Soil samples were collected with a hoe from four to five trees randomly selected in each commercial olive orchard for both taxa identification and explanatory variables data collection.*
Experimental features	*Evaluate diversity, prevalence and abundance of plant-parasitic nematodes infesting soils from cultivated olive in southern Spain.*
Data source location	*Andalusia, southern Spain. Coordinates of sampling points are provided.*
Data accessibility	*Data is provided in this data article.*
Related research article	*Archidona-Yuste A., Wiegand T., Castillo P., and Navas-Cortés J. A. Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in Southern Spain. Submitted to: Agriculture, Ecosystems and Environment*

Value of the Data

- Data may serve as benchmarks for other groups working in the field of PPN diversity infesting soils from agricultural ecosystems, and for below-ground communities and ecosystems.

- Data are based on the systematic survey with the largest sampling effort done on cultivated olive to date.
- Data show a species list of PPN attacking to cultivated olive.
- Data increase the number of PPN associated with olive trees, being estimated in about 250 species documented worldwide.
- Data provided useful information of potential factors structuring the community patterns of diversity of PPN in agricultural ecosystems.

1. Data

Data include the information of the 376 commercial olive orchards sampled, as well as the total abundance of nematodes and species richness for each commercial orchard in Table 2.1, information about the diversity of PPN found from the systematic survey performed in Table 2.2, Figures 2.1 and 2.2, and finally, information about the potential factors in structuring the community patterns of diversity of PPN detected in Tables 2.3, 2.4, 2.5, 2.6 and 2.7. A total of 48 explanatory variables were selected and related with above-ground environment, soil and agronomic management. In addition, Figure 2.1 showed the distribution of species diversity of PPN detected by classes including feeding habit and family. Finally, values of LCBD and SCBD index are provided in Tables 2.1 and 2.2, respectively. Table 2.2 showed the 27 commercial olive orchards with significant values as described by Archidona-Yuste *et al*. (2018).

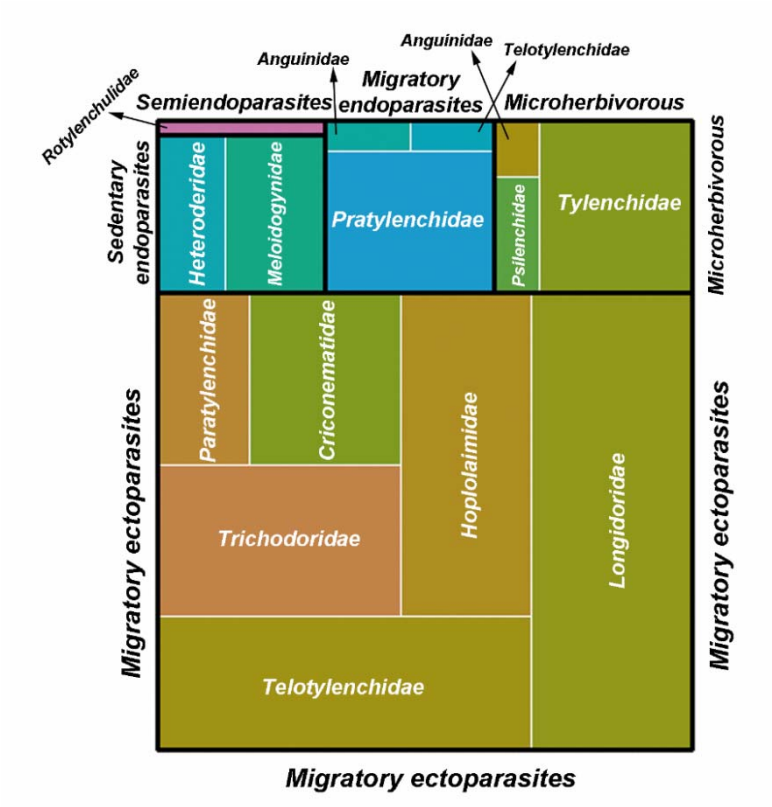


Figure 2.1: Diversity of PPN associated with cultivated olive in southern Spain. Tree map chart representing the diversity among feeding habits (black squares) and families (white chart) of PPN. The size of squares represents the number of taxa included in the feeding habit and/or family of PPN.

The diversity, prevalence and abundance of PPN associated with cultivated olive are presented in Table 2.2, Figures 2.1 and 2.2. Data were characterized by performing species diversity under integrative taxonomy identification at species level of PPN infesting soils from 376 sampled commercial olive orchards in Andalusia, southern Spain (Archidona-Yuste *et al.* 2018) (Table 2.1). Thus, 128 PPN species belonging to 38 genera and to 13 families were recorded, which highlights a high taxonomical diversity of PPN communities. However, it should be pointed out that species belonging to genus *Filenchus* were not included because of its feeding habits as plant feeding are not fully clarified (Okada *et al.* 2005).

Other PPN species such as *Heterodera avenae*, *Pratylenchus neglectus*, *Pratylenchus thornei*, *Zygotylenchus guevarai* or other species from the genera *Ditylenchus*, *Heterodera* and *Globodera* were included in the analysis although olive is not a suitable host for them but they were detected from the rhizosphere of olive tree and could be associated with host plants growing as cover crops in the orchards. The nematode abundance in each commercial olive orchard ranged from 7 to (O31) to 19,796 (O333) nematode specimens per 500 cm^3 of soil (Archidona-Yuste *et al.* 2018) (Table 2.1). The number of PPN species per nematode family ranged from one in the case of the family Rotylenchulidae to 28 species for the family Longidoridae. Other families comprising species among the most damaging plant pathogens worldwide such as Meloidogynidae encompassed six sedentary endoparasite nematodes species (*Meloidogyne* spp.). The three most prevalent families were Tylenchidae, Paratylenchidae and Criconematidae, and the nematodes families with the highest average nematode densities were Meloidogynidae, Hoplolaimidae and Paratylenchidae. In fact, migratory ectoparasite PPN such as *Helicotylenchus oleae* and *Ogma rhombosquamatum* showed the highest nematode abundance (19,720 and 9,800 nematodes per 500 cm3 of soil, respectively); however, a rare (low prevalence) of sedentary endoparasitic PPN species such as *Meloidogyne javanica* was also detected at a high nematode abundance, i.e. 10,000 nematodes per 500 cm^3 of soil. The species prevalence ranged from 0.3 (several nematodes species detected only in one sampling site) to 72.6% (*Merlinius brevidens*). Data revealed a remarkable diversity of PPN associated with olive trees, which agrees with the fact described that olive acts as host plant of a large variety of PPN (Castillo *et al.* 2010, Ali *et al.* 2014). Data increase the number of PPN associated with olive trees, being estimated in about 250 species documented worldwide (Lamberti and Vovlas 1993, Sasanelli 2009, Castillo *et al.* 2010, Ali *et al.* 2014, Palomares-Rius *et al.* 2015, Ali *et al.* 2017). The common genera of PPN observed were similar to those reported in previous surveys in olive trees in Andalusia (Palomares-Rius *et al.* 2015) and Morocco (Ali *et al.* 2017) except for the remarkable taxonomical diversity detected for the family Longidoridae (28 species), which has been already described in previous studies (Archidona-Yuste *et al.* 2016b, c, d, a). Data also showed the nematode biomass for species of PPN identified.

SCBD values ranged from almost zero to 17% for the migratory ectoparasitic PPN species *Helicotylenchus digonicus* (Table 2.2).

Figure 2.2: World cloud considering the genera of PPN associated with cultivated olive in southern Spain. The size of word indicates the number of species associated with each PPN genera.

Table 2.1 Olive orchards from cultivated olive in Andalusia (southern Spain) for detecting plant-parasitic nematodes. Olive growing areas in Andalusia have been classified into 70 biologically homogeneous zones based on environmental similarities (REDIAM 2016). Based on these zones, 376 commercial olive orchards were selected for this study. This was done in a way that the number of sampled olive orchards per biological zone was proportional to the total olive area in each zone.

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O1	**Hinojos, Huelva**	**37º15'31.6"N**	**6º22'22.4"W**	**55**	**12**	**541**	**0.0039501***
O2	Hinojos, Huelva	37º20'57.9"N	6º23'01.5"W	121	6	73	0.0033041
O3	Escacena del Campo, Huelva	37º24'06.5"N	6º22'28.0"W	130	9	177	0.0028959
O4	Villalba del Alcor, Huelva	37º20'46.0"N	6º26'29.3"W	125	8	263	0.0033954
O5	Almonte, Huelva	37º14'19.3"N	6º28'58.7"W	65	8	255	0.0035093
O6	**Villalba del Alcor, Huelva**	**37º24'03.6"N**	**6º29'46.1"W**	**96**	**8**	**717**	**0.0043700***
O7	Niebla, Huelva	37º24'04.3"N	6º42'45.1"W	101	7	98	0.0026263
O8	Niebla, Huelva	37º21'57.9"N	6º43'45.0"W	64	4	37	0.0032298
O9	Jerez de la Frontera, Cádiz	36º48'12.6"N	5º59'40.7"W	78	5	107	0.0027860
O10	Jerez de la Frontera, Cádiz	36º46'08.2"N	5º59'45.5"W	69	5	127	0.0030383
O11	Jerez de la Frontera, Cádiz	36º39'32.9"N	6º02'06.2"W	103	4	50	0.0036332
O12	Jerez de la Frontera, Cádiz	36º40'23.1"N	6º07'20.3"W	58	8	1468	0.0023036
O13	**Villaviciosa de Córdoba, Córdoba**	**38º2'52.65"N**	**5º0'43.18"W**	**494**	**10**	**630**	**0.0039054***
O14	Belmez, Córdoba	38º14'17.5"N	5º07'16.7"W	509	5	32	0.0028062
O15	Belmez, Córdoba	38º14'33.8"N	5º08'36.9"W	513	9	139	0.0029163
O16	Fuente Obejuna, Córdoba	38º17'29.3"N	5º19'16.9"W	590	8	162	0.0037636
O17	Fuente Obejuna, Córdoba	38º15'56.1"N	5º24'55.2"W	562	6	176	0.0034448
O18	La Granjuela, Córdoba	38º22'33.9"N	5º20'46.9"W	630	10	210	0.0035947
O19	La Granjuela, Córdoba	38º22'45.5"N	5º19'27.2"W	550	3	219	0.0036349
O20	Hinojosa del Duque, Córdoba	38º24'19.0"N	5º18'10.8"W	570	10	268	0.0025421
O21	Hinojosa del Duque, Córdoba	38º24'40.7"N	5º13'06.9"W	527	7	138	0.0023221
O22	El Viso, Córdoba	38º29'56.0"N	4º58'38.4"W	623	7	167	0.0015419

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O23	Alcaracejos, Córdoba	38º22'55.5"N	4º57'32.9"W	727	9	287	0.0034716
O24	Alcaracejos, Córdoba	38º15'49.0"N	4º58'47.86"W	570	8	229	0.0028375
O25	Villaharta, Córdoba	38º8'23.97"N	4º52'50.46"W	303	7	75	0.0017119
O26	Cañete de las Torres, Córdoba	37º52'31.3"N	4º20'25.1"W	341	4	136	0.0014172
O27	Porcuna, Jaén	37º52'54.3"N	4º11'29.5"W	374	6	291	0.0013965
O28	Porcuna, Jaén	37º53'44.0"N	4º08'14.3"W	229	4	187	0.0029693
O29	Andújar, Jaén	38º00'07.1"N	4º03'21.5"W	478	5	251	0.0023402
O30	Andújar, Jaén	38º07'17.0"N	3º57'41.4"W	419	6	39	0.0014365
O31	Andújar, Jaén	38º05'46.2"N	3º58'18.6"W	259	3	7	0.0024413
O32	Andújar, Jaén	38º03'49.7"N	4º00'16.7"W	191	5	149	0.0025519
O33	Marmolejo, Jaén	38º03'11.5"N	4º11'25.6"W	283	3	157	0.0027722
O34	Marmolejo, Jaén	38º03'42.0"N	4º13'24.2"W	348	4	142	0.0028993
O35	Montoro, Córdoba	38º05'59.5"N	4º16'28.3"W	452	3	28	0.0032918
O36	Montoro, Córdoba	38º07'18.9"N	4º16'44.8"W	422	7	356	0.0032150
O37	**Iznajar, Córdoba**	**37º15'39.1"N**	**4º19'20.0"W**	**448**	**8**	**140**	**0.0040370***
O38	Prado del Rey, Cádiz	36º47'17.4"N	5º33'45.00"W	89	8	133	0.0029531
O39	**Rociana del Condado, Huelva**	**37º16'45.8"N**	**6º37'20.4"W**	**539**	**4**	**213**	**0.0040512***
O40	Antequera, Málaga	37º08'36.0"N	4º31'28.8"W	212	6	199	0.0019654
O41	Antequera, Málaga	37º10'27.7"N	4º34'58.1"W	212	5	1380	0.0013928
O42	Mollina, Málaga	37º09'54.4"N	4º41'12.9"W	393	5	1589	0.0021571
O43	Antequera, Málaga	37º02'38.5"N	4º40'05.9"W	389	11	474	0.0021974
O44	Antequera, Málaga	37º01'48.9"N	4º45'30.2"W	470	8	846	0.0018290
O45	Campillos, Málaga	37º00'09.7"N	4º50'42.2"W	383	8	1444	0.0015922
O46	Ardales, Málaga	36º52'53.1"N	4º50'33.9"W	160	7	699	0.0014612
O47	Alora, Málaga	36º48'03.5"N	4º45'13.4"W	200	7	168	0.0017819
O48	Casarabonela, Málaga	36º46'13.8"N	4º46'54.7"W	368	6	461	0.0029229
O49	Casarabonela, Málaga	36º46'01.5"N	4º49'52.9"W	224	10	237	0.0023574
O50	Tolox, Málaga	36º41'58.5"N	4º52'46.6"W	317	8	448	0.0019210
O51	Monda, Málaga	36º38'13.0"N	4º48'24.2"W	409	8	515	0.0030593

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O52	Monda, Málaga	36º37'03.0"N	4º51'00.8"W	51	5	61	0.0013762
O53	Marbella, Málaga	36º30'47.0"N	4º50'02.1"W	505	10	475	0.0028430
O54	Espiel, Córdoba	38º10'58.0"N	5º2'15.31"W	566	9	550	0.0023952
O55	Espiel, Córdoba	38º10'25.7"N	5º05'01.0"W	712	11	499	0.0026249
O56	Espiel, Córdoba	38º06'28.5"N	5º04'19.5"W	729	7	401	0.0028581
O57	Espiel, Córdoba	38º03'02.7"N	4º56'49.3"W	580	7	115	0.0024678
O58	Espiel, Córdoba	37º54'17.4"N	6º16'32.1"W	531	9	1163	0.0024123
O59	Santa Olalla del Cala, Huelva	37º53'59.1"N	6º15'22.5"W	200	10	951	0.0035445
O60	Córdoba, Córdoba	37º55'22.3"N	4º45'56.5"W	430	8	173	0.0025667
O61	Montilla, Córdoba	37º32'57.5"N	4º34'11.6"W	249	3	196	0.0015881
O62	Cabra, Córdoba	37º30'55.2"N	4º29'20.7"W	177	8	479	0.0015906
O63	Cabra, Córdoba	37º29'08.9"N	4º25'55.1"W	615	8	112	0.0014784
O64	Zuheros, Córdoba	37º32'41.1"N	4º20'47.1"W	834	3	443	0.0034653
O65	Zuheros, Córdoba	37º32'06.3"N	4º18'38.3"W	647	9	705	0.0033046
O66	Zuheros, Córdoba	37º32'49.1"N	4º18'42.6"W	551	5	264	0.0014094
O67	Luque, Córdoba	37º31'03.9"N	4º12'53.6"W	421	7	518	0.0019085
O68	Luque, Córdoba	37º34'49.4"N	4º12'38.7"W	278	6	145	0.0032546
O69	Baena, Córdoba	37º40'31.8"N	4º23'39.9"W	309	5	1399	0.0035135
O70	Baena, Córdoba	37º48'50.8"N	4º18'27.5"W	295	6	129	0.0032498
O71	Alozaina, Málaga	36º43'28.9"N	4º51'06.1"W	77	6	220	0.0012781
O72	Marbella, Málaga	36º29'53.9"N	5º00'40.1"W	180	5	384	0.0018494
O73	Paterna del Campo, Huelva	37º28'50.8"N	6º29'19.5"W	810	9	454	0.0023530
O74	Alhendín, Granada	37º04'22.3"N	3º40'40.3"W	439	5	6907	0.0022341
O75	Lecrín, Granada	36º54'37.8"N	3º31'58.3"W	665	6	358	0.0028362
O76	Lanjarón, Granada	36º54'51.3"N	3º30'01.0"W	639	7	1095	0.0026383
O77	Lanjarón, Granada	36º54'27.8"N	3º27'38.2"W	611	5	306	0.0037434
O78	Lobres, Granada	36º54'41.4"N	3º12'50.5"W	717	4	78	0.0017682
O79	Torvizcón, Granada	36º53'04.0"N	3º18'14.0"W	757	5	859	0.0021787
O80	Alcolea, Almería	36º57'48.9"N	2º57'37.9"W	963	7	1418	0.0024528

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O81	Laujar de Andarax, Almería	36º58'21.4"N	2º55'22.1"W	359	4	6990	0.0023231
O82	Instinción, Almería	36º59'43.1"N	2º39'14.5"W	719	5	502	0.0029016
O83	Alicún, Almería	36º56'40.4"N	2º36'03.1"W	877	3	68	0.0014942
O84	Enix, Almería	36º52'57.8"N	2º37'28.8"W	500	6	1410	0.0021515
O85	**Tabernas, Almería**	**37º04'18.1"N**	**2º18'18.4"W**	**524**	**4**	**226**	**0.0044046***
O86	Lucainena de las Torres, Almería	37º04'50.1"N	2º11'54.3"W	500	6	4040	0.0024570
O87	Tabernas, Almería	37º05'04.7"N	2º14'33.4"W	421	6	508	0.0031701
O88	Sorbas, Almería	37º06'22.1"N	2º08'35.7"W	593	5	371	0.0023451
O89	Uleila del Campo, Almería	37º11'08.6"N	2º11'49.6"W	252	4	174	0.0021843
O90	Cádiar, Granada	36º56'54.3"N	3º07'14.5"W	329	9	279	0.0014578
O91	Úbeda, Jaén	37º55'00.4"N	3º21'05.1"W	688	11	367	0.0025861
O92	Jódar, Jaén	37º49'10.6"N	3º19'38.8"W	698	4	1352	0.0034362
O93	Bedmar y Garcíez, Jaén	37º47'57.0"N	3º22'29.8"W	706	5	423	0.0023785
O94	Belmez de la Moraleda, Jaén	37º44'43.6"N	3º21'36.4"W	814	3	226	0.0028352
O95	Huelma, Jaén	37º43'50.0"N	3º18'00.0"W	1069	3	26	0.0012897
O96	Huelma, Jaén	37º40'09.9"N	3º18'27.9"W	1019	2	37	0.0020516
O97	Cabra del Santo Cristo, Jaén	37º39'31.0"N	3º14'14.7"W	591	6	2270	0.0020565
O98	Dehesas de Guadix, Granada	37º34'16.8"N	3º04'17.0"W	1061	3	80	0.0021005
O99	Pedro Martínez, Granada	37º32'07.4"N	3º10'52.6"W	1044	6	1147	0.0021356
O100	Pedro Martínez, Granada	37º30'38.0"N	3º12'53.3"W	1041	6	1512	0.0017842
O101	Morelábor, Granada	37º28'06.3"N	3º17'20.5"W	1092	6	489	0.0016553
O102	Morelábor, Granada	37º25'51.5"N	3º20'13.5"W	1061	5	1007	0.0014642
O103	Piñar, Granada	37º23'17.0"N	3º24'47.1"W	182	5	235	0.0013437
O104	Écija, Sevilla	37º31'04.7"N	5º09'50.0"W	164	5	577	0.0022463
O105	Fuentes de Andalucía, Sevilla	37º30'21.5"N	5º24'29.3"W	169	4	47	0.0032955
O106	Carmona, Sevilla	37º29'41.6"N	5º28'33.0"W	123	5	84	0.0038680
O107	Carmona, Sevilla	37º28'37.4"N	5º42'26.7"W	139	6	1731	0.0024029
O108	Olivares, Sevilla	37º25'51.2"N	6º08'11.9"W	30	4	1666	0.0028651
O109	Sanlúcar la Mayor, Sevilla	37º23'49.9"N	6º13'20.3"W	30	4	3414	0.0021621

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O110	Sanlúcar la Mayor, Sevilla	37º23'49.0"N	6º13'19.3"W	96	8	765	0.0022083
O111	Sanlúcar la Mayor, Sevilla	37º30'50.2"N	6º12'33.5"W	56	2	50	0.0036413
O112	Guillena, Sevilla	37º33'56.2"N	6º02'33.9"W	53	3	24	0.0031674
O113	Villaverde del Río, Sevilla	37º36'22.1"N	5º54'25.5"W	395	3	937	0.0023797
O114	Villaverde del Río y Minas, Sevilla	37º39'51.4"N	5º44'56.7"W	46	7	7444	0.0038494
O115	**Lora del Río, Sevilla**	**37º37'41.0"N**	**5º40'29.4"W**	**129**	**3**	**365**	**0.0042194***
O116	**Alcolea del Río, Sevilla**	**37º37'50.5"N**	**5º35'01.4"W**	**65**	**5**	**72**	**0.0042684***
O117	**La Campana, Sevilla**	**37º35'21.4"N**	**5º27'36.4"W**	**136**	**3**	**45**	**0.0042099***
O118	**La Campana, Sevilla**	**37º36'24.0"N**	**5º23'18.9"W**	**46**	**5**	**323**	**0.0039655***
O119	**Utrera, Sevilla**	**37º09'12.2"N**	**5º46'39.9"W**	**24**	**8**	**288**	**0.0040418***
O120	Utrera, Sevilla	37º04'36.2"N	5º48'37.8"W	67	7	394	0.0017599
O121	Utrera, Sevilla	36º59'22.6"N	5º49'39.9"W	133	5	1773	0.0018613
O122	Utrera, Sevilla	36º56'37.7"N	5º48'25.2"W	45	7	2404	0.0018732
O123	Arcos de la Frontera, Cádiz	36º44'32.3"N	5º51'32.5"W	71	4	996	0.0023408
O124	Arcos de la Frontera, Cádiz	36º40'35.0"N	5º51'12.8"W	175	5	12	0.0016646
O125	Arcos de la Frontera, Cádiz	36º35'50.2"N	5º47'08.2"W	10	9	956	0.0027686
O126	**San José del Valle, Cádiz**	**36º40'44.9"N**	**5º27'11.0"W**	**140**	**8**	**611**	**0.0040427***
O127	Ubrique, Cádiz	36º52'43.0"N	5º34'31.5"W	167	5	718	0.0024564
O128	Constantina, Sevilla	37º52'56.4"N	5º38'06.7"W	138	5	60	0.0024440
O129	Santa Cruz, Córdoba	37º57'37.2"N	4º27'56.8"W	135	5	709	0.0027089
O130	Ecija, Sevilla	37º31'45.9"N	4º57'52.6"W	140	6	845	0.0023221
O131	Maro, Málaga	36º44'50.5"N	3º47'12.1"W	108	7	436	0.0025161
O132	Velez Málaga, Málaga	36º46'15.8"N	4º05'22.9"W	73	7	143	0.0023267
O133	Canillas de Aceituno, Málaga	36º50'41.5"N	4º07'29.7"W	172	4	529	0.0020247
O134	Alcaucín, Málaga	36º56'27.8"N	4º07'36.4"W	678	5	247	0.0021700
O135	Alfarnate, Málaga	37º00'35.3"N	4º13'55.9"W	963	5	116	0.0026151
O136	Loja, Granada	37º03'14.7"N	4º14'47.9"W	267	6	235	0.0026386
O137	Loja, Granada	37º08'40.0"N	4º12'51.9"W	177	4	939	0.0022364
O138	**Castillo de las Guardas, Sevilla**	**37º42'39.2"N**	**6º18'45.5"W**	**301**	**8**	**98**	**0.0039617***

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O139	Higuera de la Sierra, Huelva	37º50'36.0"N	6º27'32.2"W	636	7	1085	0.0038587
O140	Aracena, Huelva	37º52'15.0"N	6º30'30.1"W	585	5	98	0.0033139
O141	Aracena, Huelva	37º53'10.2"N	6º33'10.5"W	694	6	59	0.0033554
O142	Fuenteheridos, Huelva	37º55'17.7"N	6º40'03.5"W	657	6	263	0.0030968
O143	Cortegana, Huelva	37º55'40.2"N	6º49'27.1"W	664	5	59	0.0032660
O144	Aroche, Huelva	37º55'45.5"N	6º57'11.8"W	526	5	150	0.0036269
O145	Rosal de la Frontera, Huelva	37º58'32.5"N	7º11'18.5"W	208	6	230	0.0020732
O146	Rosal de la Frontera, Huelva	37º57'52.7"N	7º14'03.5"W	220	8	159	0.0026179
O147	Mengíbar, Jaén	38º1'21.70"N	3º46'38.68"W	279	5	382	0.0022702
O148	Luque, Córdoba	37º34'30.9"N	4º10'37.8"W	443	6	164	0.0030759
O149	Jerez de la Frontera, Cádiz	36º48'10.3"N	6º10'25.5"W	53	10	422	0.0035536
O150	La Rambla, Córdoba	37º37'24.3"N	4º42'17.3"W	366	8	117	0.0035630
O151	La Rambla, Córdoba	37º37'42.6"N	4º42'50.2"W	377	7	202	0.0021412
O152	Marchena, Sevilla	37º20'19.7"N	5º18'27.6"W	141	7	281	0.0014025
O153	Marchena, Sevilla	37º18'28.2"N	5º23'38.1"W	151	14	196	0.0028052
O154	Riogordo,Málaga	36º55'20.3"N	4º17'10.0"W	437	9	224	0.0023030
O155	**Tabernas, Almería**	**37º06'19.5"N**	**2º17'09.9"W**	**549**	**6**	**980**	**0.0041281***
O156	Albaricoques-Níjar, Almería	36º51'26.6"N	2º06'08.1"W	165	5	49	0.0038506
O157	Coto Ríos, Jaén	38º01'49.4"N	2º52'05.2"W	687	10	309	0.0021103
O158	Santa Mª de Trassierra, Córdoba	37º54'46.0"N	4º50'53.8"W	437	8	103	0.0037143
O159	Castillo de Locubín, Jaén	37º34'28.6"N	3º58'59.9"W	718	6	169	0.0017382
O160	Morón de la Frontera, Sevilla	37º05'16.0"N	5º30'38.4"W	193	6	913	0.0027212
O161	Pozo Alcón, Jaén	37º43'47.6"N	2º55'18.0"W	948	3	1173	0.0034160
O162	Santa Mª de Trassierra, Córdoba	37º55'12.4"N	4º52'33.5"W	558	9	434	0.0022608
O163	Córdoba, Córdoba	37º55'18.8"N	4º45'56.4"W	197	11	390	0.0024856
O164	Bedmar y Garcíez, Jaén	37º47'52.1"N	3º22'22.6"W	720	5	3089	0.0038138
O165	Nueva Carteya, Córdoba	37º35'49.6"N	4º29'42.6"W	384	6	177	0.0034460
O166	**Cabra, Córdoba**	**37º30'35.9"N**	**4º31'30.9"W**	**414**	**6**	**2379**	**0.0042721***
O167	Espejo, Córdoba	37º40'19.9"N	4º32'39.8"W	331	6	139	0.0021533

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O168	Lucena, Córdoba	37º26'18.7"N	4º32'35.5"W	421	8	164	0.0028766
O169	Montemayor, Córdoba	37º40'40.4"N	4º41'03.5"W	251	6	48	0.0026783
O170	La Rambla, Córdoba	37º39'24.4"N	4º46'29.1"W	245	5	245	0.0026092
O171	Montalbán de Córdoba, Córdoba	37º34'01.8"N	4º46'19.9"W	169	4	231	0.0028817
O172	Santaella, Córdoba	37º29'58.0"N	4º46'54.0"W	240	5	4342	0.0030187
O173	**Puente Genil, Córdoba**	**37º23'02.0"N**	**4º45'54.5"W**	**212**	**5**	**7599**	**0.0039211***
O174	**Gibraleón, Huelva**	**37º20'40.4"N**	**7º02'05.1"W**	**59**	**10**	**10071**	**0.0040633***
O175	**Gibraleón, Huelva**	**37º24'12.4"N**	**7º00'41.1"W**	**73**	**5**	**38**	**0.0042649***
O176	Trigueros, Huelva	37º23'38.1"N	6º49'57.1"W	91	9	470	0.0023190
O177	Trigueros, Huelva	37º21'57.6"N	6º49'04.9"W	61	8	79	0.0022065
O178	Niebla, Huelva	37º21'54.1"N	6º39'06.4"W	59	13	176	0.0034490
O179	La Palma del Condado, Huelva	37º23'16.9"N	6º34'49.4"W	99	6	37	0.0027454
O180	**Hinojos, Huelva**	**37º18'16.5"N**	**6º22'24.4"W**	**83**	**10**	**10605**	**0.0041028***
O181	Hinojos, Huelva	37º18'25.8"N	6º20'37.8"W	73	7	949	0.0034245
O182	Ecija, Sevilla	37º35'42.6"N	4º58'26.8"W	234	5	65	0.0022291
O183	Marchena, Sevilla	37º20'26.6"N	5º18'23.5"W	140	7	281	0.0014025
O184	La Puebla de Cazalla, Sevilla	37º14'08.8"N	5º15'30.8"W	183	5	105	0.0033053
O185	Osuna, Sevilla	37º13'57.0"N	5º10'49.2"W	217	6	253	0.0026018
O186	Osuna, Sevilla	37º12'35.6"N	5º07'55.0"W	266	4	202	0.0029392
O187	Osuna, Sevilla	37º09'10.0"N	5º06'37.5"W	466	7	1976	0.0017018
O188	El Saucejo, Sevilla	37º05'44.2"N	5º05'30.0"W	501	5	2210	0.0017913
O189	El Saucejo, Sevilla	37º05'01.7"N	5º06'53.4"W	464	5	668	0.0037886
O190	Córdoba, Córdoba	37º45'24.0"N	4º49'49.8"W	283	8	1075	0.0013125
O191	Santaella, Córdoba	37º30'40.3"N	4º51'10.1"W	176	5	250	0.0017708
O192	Lopera, Jaén	37º59'08.4"N	4º15'13.0"W	195	8	106	0.0022066
O193	Andújar, Jaén	38º02'24.9"N	3º58'02.4"W	216	6	6337	0.0033242
O194	Guarromán, Jaén	38º10'54.5"N	3º42'04.7"W	351	4	53	0.0024257
O195	Ibros, Jaén	38º04'19.1"N	3º33'16.3"W	345	8	1183	0.0016047
O196	Jabalquinto, Jaén	38º01'18.8"N	3º46'27.5"W	315	9	474	0.0015316

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O197	Bailén, Jaén	38º02'15.2"N	3º48'09.0"W	266	5	729	0.0014233
O198	Torre del Campo, Jaén	37º48'49.9"N	3º51'46.5"W	480	7	228	0.0027844
O199	Torredonjimeno, Jaén	37º45'16.7"N	4º07'08.3"W	381	8	1305	0.0017618
O200	El Carpio, Córdoba	37º57'07.2"N	4º30'11.1"W	137	5	1128	0.0022047
O201	Pedro Abad, Córdoba	37º58'15.6"N	4º26'19.4"W	173	6	27	0.0032630
O202	Montoro, Córdoba	38º00'27.0"N	4º17'52.4"W	167	6	97	0.0018952
O203	Montoro, Córdoba	38º01'52.1"N	4º20'19.7"W	259	6	135	0.0022104
O204	Castro del Río, Córdoba	37º41'19.3"N	4º24'12.6"W	307	5	323	0.0022815
O205	Baena, Córdoba	37º41'24.8"N	4º21'03.5"W	312	4	429	0.0024532
O206	Castro del Río, Córdoba	37º40'13.9"N	4º30'03.3"W	327	9	1635	0.0024477
O207	Moriles, Córdoba	37º25'01.4"N	4º38'29.9"W	308	5	69	0.0021067
O208	Alameda, Málaga	37º13'03.1"N	4º42'22.7"W	448	8	197	0.0022599
O209	Antequera, Málaga	37º05'37.8"N	4º33'52.4"W	435	6	324	0.0021843
O210	Antequera, Málaga	37º00'13.0"N	4º35'18.1"W	649	5	56	0.0019845
O211	Alora, Málaga	36º52'43.0"N	4º40'59.0"W	241	4	75	0.0030859
O212	Colmenar,Málaga	36º54'20.0"N	4º21'12.8"W	667	4	93	0.0036512
O213	Riogordo, Málaga	36º55'27.5"N	4º17'08.0"W	495	8	578	0.0020036
O214	La Tres Villas, Almería	37º08'55.4"N	2º43'30.9"W	763	9	702	0.0033295
O215	**La Tres Villas, Almería**	**37º08'15.1"N**	**2º43'28.1"W**	**706**	**7**	**1762**	**0.0042730***
O216	Tabernas, Almería	37º06'07.1"N	2º16'41.7"W	533	3	121	0.0037587
O217	**Uleila del Campo, Almería**	**37º09'13.2"N**	**2º12'16.1"W**	**572**	**4**	**739**	**0.0041255***
O218	**Sorbas, Almería**	**37º08'52.2"N**	**2º09'23.2"W**	**490**	**6**	**934**	**0.0043722***
O219	Huércal-Overa, Almería	37º19'18.7"N	1º58'18.8"W	224	4	79	0.0024104
O220	Purchena, Almería	37º21'59.5"N	2º20'56.9"W	543	3	43	0.0033200
O221	Urrácal, Almería	37º22'30.3"N	2º21'34.3"W	592	9	507	0.0032935
O222	Armiña de Almanzora, Almería	37º21'39.2"N	2º25'33.7"W	629	3	684	0.0030571
O223	Serón, Almería	37º22'07.0"N	2º29'33.5"W	785	4	233	0.0022217
O224	Baza, Granada	37º33'00.5"N	2º44'34.5"W	700	6	501	0.0033141
O225	Baza, Granada	37º34'32.2"N	2º46'13.0"W	686	7	764	0.0030934

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O226	Cortes y Graena, Granada	37º17'52.5"N	3º13'05.3"W	972	5	92	0.0015521
O227	Diezma, Granada	37º19'15.9"N	3º21'07.9"W	1282	5	64	0.0024681
O228	Alfacar, Granada	37º14'26.2"N	3º34'59.3"W	880	7	618	0.0022440
O229	Güevéjar, Granada	37º15'30.7"N	3º36'13.4"W	849	9	115	0.0022766
O230	Pinos Puente, Granada	37º11'51.2"N	3º52'19.9"W	530	5	220	0.0024649
O231	Jerez de la Frontera, Cádiz	36º44'51.4"N	6º00'24.4"W	32	7	351	0.0024601
O232	Jerez de la Frontera, Cádiz	36º46'21.3"N	5º56'52.0"W	73	4	243	0.0035819
O233	San José del Valle, Cádiz	36º34'37.9"N	5º49'15.2"W	218	5	735	0.0013023
O234	Algar, Cádiz	36º40'00.8"N	5º38'56.2"W	211	8	897	0.0025868
O235	Zahara de la Sierra, Cádiz	36º50'51.6"N	5º23'58.6"W	390	7	476	0.0028059
O236	Aldodonales, Cádiz	36º51'59.9"N	5º24'42.2"W	306	10	889	0.0024796
O237	Ecija, Sevilla	37º39'19.4"N	4º58'00.0"W	201	5	1050	0.0023624
O238	Ecija, Sevilla	37º40'31.4"N	4º59'02.9"W	183	6	143	0.0019782
O239	Marchena, Sevilla	37º16'46.4"N	5º21'51.1"W	150	5	488	0.0022139
O240	Marchena, Sevilla	37º16'05.1"N	5º21'34.1"W	148	5	188	0.0018296
O241	Marchena, Sevilla	37º15'00.8"N	5º22'09.4"W	162	7	157	0.0027083
O242	La Puebla de Cazalla, Sevilla	37º12'54.0"N	5º19'19.2"W	189	8	205	0.0032287
O243	Gibraleón, Huelva	37º21'16.7"N	7º01'15.3"W	58	7	233	0.0027071
O244	Gibraleón, Huelva	37º22'01.0"N	7º00'45.9"W	60	3	267	0.0013664
O245	Gibraleón, Huelva	37º23'22.5"N	6º55'50.0"W	67	5	312	0.0013573
O246	Beas, Huelva	37º25'06.1"N	6º47'09.8"W	109	6	318	0.0014778
O247	Beas, Huelva	37º24'09.8"N	6º45'44.8"W	82	4	132	0.0022761
O248	Beas, Huelva	37º23'33.0"N	6º44'57.8"W	67	6	177	0.0021055
O249	**Bollullos par del Condado, Huelva**	**37º19'22.7"N**	**6º32'47.8"W**	**101**	**6**	**1027**	**0.0043603***
O250	Espiel, Córdoba	38º09'24.2"N	5º05'59.5"W	547	10	217	0.0025397
O251	San José de la Rinconada, Sevilla	37º26'18.0"N	5º50'21.4"W	41	6	156	0.0032287
O252	Huévar del Aljarafe, Sevilla	37º21'49.0"N	6º17'24.1"W	67	9	321	0.0030440
O253	Huévar del Aljarafe, Sevilla	37º21'07.1"N	6º17'45.8"W	117	5	431	0.0015883
O254	Aznalcázar, Sevilla	37º17'34.5"N	6º16'49.5"W	40	12	17481	0.0023603

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O255	Bollullos de la Mitación, Sevilla	37º19'48.2"N	6º08'53.9"W	81	7	1362	0.0037966
O256	**Dos Hermanas, Sevilla**	**37º14'59.4"N**	**5º55'30.5"W**	**45**	**9**	**330**	**0.0039641***
O257	Dos Hermanas, Sevilla	37º12'50.2"N	5º55'57.3"W	24	8	259	0.0016299
O258	El Pinar, Granada	36º54'45.8"N	3º33'56.4"W	759	4	1310	0.0017594
O259	El Valle, Granada	36º55'01.6"N	3º34'34.0"W	722	6	484	0.0022285
O260	Vegas del Genil, Granada	37º09'27.8"N	3º43'51.1"W	626	8	1269	0.0025785
O261	Las Gabias, Granada	37º08'17.5"N	3º44'05.2"W	663	4	335	0.0025266
O262	Alhama de Granada, Granada	37º08'30.4"N	3º58'40.6"W	653	7	328	0.0022782
O263	Santa Cruz del Comercio, Granada	37º04'44.6"N	3º59'31.4"W	745	7	349	0.0034424
O264	Loja, Granada	37º12'53.4"N	4º04'45.2"W	525	8	372	0.0031186
O265	Loja, Granada	37º14'12.1"N	4º04'43.9"W	543	2	33	0.0033295
O266	Utrera, Sevilla	37º13'22.6"N	5º49'08.0"W	55	12	672	0.0031654
O267	Utrera, Sevilla	37º13'48.3"N	5º49'21.7"W	59	6	541	0.0035660
O268	Utrera, Sevilla	37º06'34.5"N	5º40'27.6"W	112	5	384	0.0028069
O269	Utrera, Sevilla	37º06'36.5"N	5º40'36.1"W	86	9	257	0.0027464
O270	Utrera, Sevilla	37º07'17.3"N	5º38'08.1"W	98	6	1475	0.0029147
O271	El Arahal, Sevilla	37º11'34.3"N	5º34'15.5"W	97	6	1394	0.0022191
O272	Morón de la Frontera, Sevilla	37º07'27.0"N	5º30'15.5"W	174	6	989	0.0031236
O273	Montellano, Sevilla	37º02'28.3"N	5º33'23.4"W	196	12	1182	0.0033468
O274	Olvera, Cádiz	36º56'24.7"N	5º20'05.8"W	304	4	713	0.0033820
O275	Posadas, Córdoba	37º48'47.3"N	5º06'36.1"W	125	2	1718	0.0034729
O276	Hornachuelos, Córdoba	37º49'08.1"N	5º11'43.9"W	124	3	52	0.0035009
O277	Hornachuelos, Córdoba	37º48'00.0"N	5º14'04.6"W	85	3	62	0.0033027
O278	Peñaflor, Sevilla	37º45'01.5"N	5º19'41.1"W	170	6	156	0.0034573
O279	La Puebla de los Infantes, Sevilla	37º46'34.7"N	5º21'24.6"W	242	9	928	0.0028052
O280	La Puebla de los Infantes, Sevilla	37º47'04.5"N	5º22'21.3"W	202	10	1053	0.0018604
O281	La Puebla de los Infantes, Sevilla	37º46'39.9"N	5º23'09.2"W	262	7	1138	0.0029147
O282	La Puebla de los Infantes, Sevilla	37º46'42.7"N	5º22'01.5"W	228	4	1489	0.0016859
O283	Constantina, Sevilla	37º45'08.0"N	5º34'38.6"W	359	8	1446	0.0033929

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O284	**Fuente Palmera, Córdoba**	**37º43'21.4"N**	**5º07'48.8"W**	**133**	**4**	**2463**	**0.0044077***
O285	El Saucejo, Sevilla	37º03'11.7"N	5º04'35.2"W	580	3	110	0.0013670
O286	Alcalá del Valle, Cádiz	36º56'25.6"N	5º08'35.1"W	751	4	1112	0.0029298
O287	Alcalá del Valle, Cádiz	36º56'27.9"N	5º08'08.0"W	754	6	233	0.0027890
O288	Alcalá del Valle, Cádiz	36º53'22.8"N	5º10'58.2"W	618	7	511	0.0018271
O289	Setenil de las Bodegas, Cádiz	36º52'34.5"N	5º09'23.5"W	649	9	973	0.0024214
O290	Setenil de las Bodegas, Cádiz	36º50'48.4"N	5º13'11.5"W	776	5	183	0.0029374
O291	Ronda, Málaga	36º43'32.7"N	5º10'19.8"W	751	9	440	0.0031657
O292	Ronda, Málaga	36º47'45.7"N	5º06'23.2"W	769	5	72	0.0032048
O293	Córdoba, Córdoba	37º52'16.9"N	4º42'53.5"W	119	9	74	0.0035676
O294	Córdoba, Córdoba	37º52'45.1"N	4º42'17.2"W	103	4	224	0.0030276
O295	Adamuz, Córdoba	38º00'30.1"N	4º32'17.1"W	225	8	620	0.0025475
O296	Adamuz, Córdoba	38º03'15.6"N	4º33'01.0"W	430	7	162	0.0026257
O297	Adamuz, Córdoba	38º04'55.2"N	4º31'40.0"W	373	3	1752	0.0034213
O298	Adamuz, Córdoba	38º01'01.4"N	4º30'50.4"W	221	2	46	0.0032216
O299	Linares, Jaén	38º05'52.8"N	3º40'36.0"W	347	5	724	0.0033412
O300	Linares, Jaén	38º06'57.8"N	3º35'49.5"W	447	6	662	0.0032065
O301	Linares, Jaén	38º08'11.2"N	3º32'59.0"W	328	2	60	0.0033533
O302	Vilchez, Jaén	38º08'47.3"N	3º31'31.6"W	308	5	4232	0.0032984
O303	Arquillos, Jaén	38º11'13.0"N	3º25'26.6"W	393	4	606	0.0014655
O304	Navas de San Juan, Jaén	38º11'02.9"N	3º21'33.8"W	511	3	1136	0.0022111
O305	Úbeda, Jaén	38º07'55.0"N	3º21'33.3"W	370	5	114	0.0016853
O306	Úbeda, Jaén	38º04'04.3"N	3º13'25.8"W	723	5	134	0.0020831
O307	Sabiote, Jaén	38º05'30.0"N	3º09'46.6"W	666	4	1426	0.0014770
O308	Iznatoraf, Jaén	38º08'42.1"N	3º01'57.8"W	826	4	1572	0.0029243
O309	Beas de Segura, Jaén	38º16'11.3"N	2º57'41.1"W	532	6	1051	0.0020722
O310	Arroyo del Ojanco, Jaén	38º17'51.2"N	2º56'15.7"W	520	6	189	0.0018165
O311	Génave, Jaén	38º25'55.9"N	2º43'26.3"W	867	4	91	0.0021150
O312	Génave, Jaén	38º26'43.8"N	2º41'36.4"W	1104	4	48	0.0026945

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O313	Benatae, Jaén	38º22'07.9"N	2º41'15.1"W	634	9	120	0.0016854
O314	La Iruela, Jaén	37º56'35.0"N	2º57'27.1"W	925	5	690	0.0028353
O315	Quesada, Jaén	37º50'36.2"N	3º05'28.2"W	836	6	196	0.0019142
O316	Huesa, Jaén	37º44'23.0"N	3º04'50.4"W	464	6	226	0.0018841
O317	Hinojares, Jaén	37º43'04.3"N	2º58'48.5"W	773	4	850	0.0033486
O318	Pozo Alcón, Jaén	37º44'01.5"N	2º55'19.8"W	981	3	77	0.0027161
O319	Castril,Granada	37º47'45.6"N	2º52'17.1"W	1109	7	839	0.0033614
O320	Huéscar, Granada	37º48'43.3"N	2º35'06.2"W	956	7	302	0.0031865
O321	Huéscar, Granada	37º50'20.9"N	2º31'58.4"W	988	5	1390	0.0019246
O322	Prado del Rey, Cádiz	36º46'44.6"N	5º33'31.3"W	356	6	491	0.0024043
O323	Fernán Núñez, Córdoba	37º41'44.0"N	4º44'54.5"W	251	4	881	0.0022497
O324	Lucena, Córdoba	37º24'11.4"N	4º31'47.1"W	404	11	1677	0.0024988
O325	Lucena, Córdoba	37º21'52.1"N	4º29'22.6"W	529	8	642	0.0024923
O326	Rute, Córdoba	37º23'28.1"N	4º24'49.9"W	630	7	323	0.0025106
O327	Rute, Córdoba	37º21'48.3"N	4º24'48.8"W	519	9	1546	0.0019566
O328	Iznájar, Córdoba	37º19'10.4"N	4º18'16.9"W	635	9	717	0.0017413
O329	Iznájar, Córdoba	37º16'07.1"N	4º18'26.6"W	461	10	359	0.0034249
O330	Iznájar, Córdoba	37º17'25.5"N	4º16'42.8"W	514	6	310	0.0014768
O331	Algarinejo, Granada	37º19'46.1"N	4º14'08.7"W	794	6	361	0.0027775
O332	Priego de Córdoba, Córdoba	37º25'37.0"N	4º12'19.2"W	751	7	210	0.0022001
O333	Lucena, Córdoba	37º34'09.7"N	4º13'09.8"W	451	5	19796	0.0035227
O334	Lucena, Córdoba	37º33'58.3"N	4º13'13.9"W	459	5	355	0.0029172
O335	Alcaudete, Jaén	37º35'44.6"N	4º07'57.7"W	501	6	276	0.0033196
O336	Alcaudete, Jaén	37º34'54.9"N	4º06'15.0"W	569	5	745	0.0033213
O337	Alcalá la Real, Jaén	37º27'23.9"N	3º56'36.9"W	881	8	742	0.0029092
O338	Alcalá la Real, Jaén	37º27'15.7"N	3º53'03.2"W	889	5	1551	0.0020559
O339	Alcalá la Real, Jaén	37º26'36.6"N	3º49'46.2"W	911	5	848	0.0030853
O340	Colomera, Granada	37º25'52.9"N	3º42'34.1"W	898	7	859	0.0033901
O341	Benalúa de las Villas, Granada	37º26'55.7"N	3º38'54.0"W	871	6	267	0.0021660

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O342	Noalejo, Jaén	37º30'45.3"N	3º38'13.2"W	971	4	126	0.0027478
O343	Noalejo, Jaén	37º32'59.0"N	3º38'30.1"W	899	5	1168	0.0028103
O344	Cambil, Jaén	37º38'41.1"N	3º36'42.6"W	671	4	745	0.0033561
O345	Cambil, Jaén	37º38'32.3"N	3º36'06.0"W	767	3	979	0.0020844
O346	Pegalajar, Jaén	37º43'28.3"N	3º40'09.0"W	577	5	977	0.0020730
O347	Herrera, Sevilla	37º20'08.7"N	4º51'15.5"W	306	4	134	0.0016900
O348	Marinaleda, Sevilla	37º18'43.9"N	4º53'05.1"W	421	5	987	0.0023478
O349	Aguadulce, Sevilla	37º15'52.0"N	4º57'31.9"W	346	5	734	0.0023742
O350	Gilena, Sevilla	37º15'27.4"N	4º55'58.8"W	428	5	537	0.0027607
O351	Martín de la Jara, Sevilla	37º07'38.7"N	4º57'00.9"W	430	4	1988	0.0031195
O352	Sierra de Yeguas, Málaga	37º07'39.6"N	4º54'39.6"W	441	4	1197	0.0017748
O353	Campillos, Málaga	37º05'26.3"N	4º52'03.3"W	491	4	144	0.0027126
O354	Bobadilla, Málaga	37º03'11.8"N	4º45'13.6"W	412	6	505	0.0035049
O355	Bujalance, Córdoba	37º54'20.4"N	4º25'00.8"W	251	4	176	0.0018113
O356	Arjona, Jaén	37º56'04.2"N	4º04'36.4"W	403	5	151	0.0023032
O357	Montilla, Córdoba	37º34'03.8"N	4º36'32.5"W	339	9	597	0.0021325
O358	Cabra, Córdoba	37º33'17.0"N	4º30'25.5"W	550	8	406	0.0020063
O359	Cabra, Córdoba	37º30'45.8"N	4º24'40.5"W	558	9	316	0.0017762
O360	Luque, Córdoba	37º32'31.7"N	4º16'05.7"W	660	10	880	0.0017256
O361	Baena, Córdoba	37º42'29.2"N	4º21'31.1"W	381	3	164	0.0021307
O362	Córdoba, Córdoba	37º51'33.8"N	4º21'58.8"W	316	7	479	0.0024839
O363	Paterna del Campo, Huelva	37º28'32.3"N	6º25'03.4"W	122	10	283	0.0026974
O364	Paterna del Campo, Huelva	37º28'50.3"N	6º29'59.9"W	190	7	2152	0.0027154
O365	Dúrcal, Granada	37º01'01.4" N	3º34'30.1"W	904	2	168	0.0020912
O366	Nigüelas, Granada	36º58'09.8"N	3º32'30.8"W	828	5	79	0.0022904
O367	**Ugíjar, Granada**	**36º58'21.0"N**	**3º00'43.4"W**	**512**	**4**	**57**	**0.0040525***
O368	Padules, Almería	37º00'06.2"N	2º47'01.4"W	792	6	788	0.0012290
O369	**Alhabia, Almería**	**36º59'02.7"N**	**2º35'15.9"W**	**267**	**3**	**854**	**0.0039660***
O370	Tabernas, Almería	37º04'56.6"N	2º17'11.4"W	522	6	854	0.0035642

Olive orchard code	Locality, province	Latitude	Longitude	Altitude[1]	Richness	Abundance	LCBD[2]
O371	Úbeda, Jaén	37º59'51.3"N	3º22'57.5"W	637	4	153	0.0016660
O372	Úbeda, Jaén	37º57'45.9"N	3º19'15.9"W	389	5	576	0.0026984
O373	Jódar, Jaén	37º47'47.5"N	3º21'20.1"W	790	3	277	0.0018790
O374	Cabra del Santo Cristo, Jaén	37º39'29.5"N	3º16'40.5"W	1041	4	304	0.0023654
O375	Alicún de Ortega, Granada	37º37'21.1"N	3º08'50.3"W	710	4	128	0.0021548
O376	Morelábor, Granada	37º27'47.1"N	3º17'17.6"W	1017	7	901	0.0029735

[1] Mean altitude measured at the scale of the olive orchard in meters.

[2] LCBD: Local Contribution to Beta Diversity. Values in bold with * note significant contribution to beta diversity ($P < 0.05$) according to Legendre and De Cáceres (2013).

Table 2.2 Plant-parasitic nematode species identified in cultivated olive in Andalusia (southern Spain).

Nematode species[1]	Species code	Feeding habits[2]	Prevalence (%)[3]	Nematode abundance			Biomass[4]	SCBD[5]
				Mean	Min	Max		
1. *Aglenchus agricola*	S001	microherviborous	17.8	12.9	2	74	0.091	0.002125
2. *Amplimerlinius icarus*	S002	migratory ectoparasite	3.5	17.1	2	56	3.095	0.010993
3. *Amplimerlinius magnistylus*	S003	migratory ectoparasite	0.3	3	3	3	3.292	0.000136
4. *Amplimerlinius paraglobigerus*	S004	migratory ectoparasite	1.1	4	2	6	0.350	0.000810
5. *Aorolaimus perscitus*	S007	migratory ectoparasite	4.2	27	1	287	0.755	0.006878
6. *Aorolaimus* sp.	S008	migratory ectoparasite	0.3	8	8	8	0.755	0.000026
7. *Basiria* sp.	S010	microherviborous	1.3	13.4	2	49	0.168	0.000322
8. *Bitylenchus hispaniensis*	S011	migratory ectoparasite	13.0	51.0	3	612	0.196	0.011619
9. *Bitylenchus maximus*	S012	migratory ectoparasite	0.5	49	2	96	0.667	0.001768
10. *Coslenchus alacinatus*	S013	microherviborous	3.2	6.7	3	12	0.099	0.000312
11. *Coslenchus costatus*	S014	microherviborous	4.8	12.2	3	34	0.107	0.001139
12. *Coslenchus indicus*	S015	microherviborous	0.3	3	3	3	0.108	0.000002
13. *Criconema annuliferum*	S016	migratory ectoparasite	10.1	29.8	1	224	0.943	0.016206
14. *Criconemoides informis*	S019	migratory ectoparasite	20.5	19.4	2	181	0.608	0.017114
15. *Criconemoides morgensis*	S020	migratory ectoparasite	0.3	210	210	210	0.740	0.001339
16. *Criconemoides sphaerocephalum*	S023	migratory ectoparasite	2.7	10.6	1	28	0.317	0.000773
17. *Criconemoides xenoplax*	S024	migratory ectoparasite	5.6	103.3	2	924	0.813	0.015797

Nematode species[1]	Species code	Feeding habits[2]	Prevalence (%)[3]	Nematode abundance			Biomass[4]	SCBD[5]
				Mean	Min	Max		
18. *Ditylenchus dipsaci* [6]	S029	migratory endoparasite	4	4.5	1	14	1.320	0.002969
19. *Ditylenchus* sp. [6]	S031	microherviborous	10.9	4	1	12	0.588	0.006853
20. *Dolichorhynchus lamelliferus*	S033	migratory ectoparasite	0.3	7	7	7	0.705	0.000006
21. *Dolichorhynchus parvus*	S035	migratory ectoparasite	1.6	126	1	506	0.091	0.003372
22. *Dolichorhynchus* sp 1	S036	migratory ectoparasite	2.1	35.4	3	112	0.499	0.004466
23. *Dolichorhynchus* sp 3	S038	migratory ectoparasite	0.3	38	38	38	0.487	0.000774
24. *Globodera* sp. * [6]	S049	sedentary endoparasite	0.3	1	1	1	0.114	0.000002
25. *Gracilacus steineri*	S051	migratory ectoparasite	1.6	28	5	71	0.033	0.000084
26. *Gracilacus straeleni*	S052	migratory ectoparasite	0.5	21	11	31	0.054	0.000081
27. *Helicotylenchus canadensis*	S053	migratory ectoparasite	1.6	337.3	14	1964	0.586	0.011342
28. *Helicotylenchus digonicus*	S054	migratory ectoparasite	48.1	485.3	2	7120	0.247	0.171107
29. *Helicotylenchus exallus*	S055	migratory ectoparasite	2.4	37.3	12	119	0.244	0.002366
30. *Helicotylenchus microlobus*	S056	migratory ectoparasite	2.4	710	14	3076	0.336	0.018585
31. *Helicotylenchus minzi*	S057	migratory ectoparasite	0.5	23	13	33	0.272	0.002041
32. *Helicotylenchus oleae*	S058	migratory ectoparasite	20.2	599.9	7	19720	0.145	0.079150
33. *Helicotylenchus* sp 1	S060	migratory ectoparasite	0.3	2312	2312	2312	0.347	0.003498
34. *Helicotylenchus* sp 4	S063	migratory ectoparasite	0.3	4	4	4	0.333	0.000327
35. *Helicotylenchus* sp 5	S064	migratory ectoparasite	0.3	14	14	14	0.326	0.000430
36. *Helicotylenchus vulgaris*	S065	migratory ectoparasite	18.9	268	4	1092	0.662	0.116413
37. *Hemicriconemoides macrodorus*	S066	migratory ectoparasite	2.1	45.7	1	112	0.714	0.006267

Nematode speces[1]	Species code	Fedding habits[2]	Prevalence (%)[3]	Nematode abundance Mean	MIn	Max	Biomass[4]	SCBD[5]
38. *Hemicycliophora iberica*	S070	migratory ectoparasite	0.3	2	2	2	0.572	0.000099
39. *Heterodera avenae** [6]	S077	sedentary endoparasite	2.4	43.5	2	345	0.154	0.000161
40. *Heterodera mediterranea**	S081	sedentary endoparasite	1.6	22.3	5	46	0.104	0.002558
41. *Heterodera* sp. * [6]	S083	sedentary endoparasite	0.3	4	4	4	0.102	0.000026
42. *Longidorus alvegus*	S087	migratory ectoparasite	0.5	6.5	1	12	6.302	0.002652
43. *Longidorus indalus*	S091	migratory ectoparasite	1.6	11.2	1	52	3.794	0.005117
44. *Longidorus macrodorus*	S261	migratory ectoparasite	0.3	1	1	1	72.799	0.003572
45. *Longidorus magnus*	S095	migratory ectoparasite	0.8	1.7	1	2	72.453	0.003382
46. *Longidorus oleae*	S096	migratory ectoparasite	0.5	2.5	2	3	37.027	0.000489
47. *Longidorus rubi*	S098	migratory ectoparasite	0.3	2	2	2	52.429	0.000256
48. *Longidorus vineacola*	S104	migratory ectoparasite	0.3	1	1	1	17.890	0.003057
49. *Longidorus vinearum*	S105	migratory ectoparasite	0.3	3	3	3	58.502	0.001073
50. *Longidorus wicuolea*	S106	migratory ectoparasite	0.3	5	5	5	28.861	0.000842
51. *Meloidogyne arenaria**	S107	sedentary endoparasite	0.8	59.7	2	138	0.068	0.000024
52. *Meloidogyne artiellia**[6]	S108	sedentary endoparasite	0.5	11.5	9	14	0.054	0.000002
53. *Meloidogyne hapla**	S110	sedentary endoparasite	0.3	2	2	2	0.088	0.000002
54. *Meloidogyne incognita**	S112	sedentary endoparasite	0.8	1951.7	4	5727	0.049	0.003219
55. *Meloidogyne javanica**	S113	sedentary endoparasite	3.7	781.4	1	10000	0.068	0.006666
56. *Meloidogyne oleae* *	S114	sedentary endoparasite	0.5	25	4	46	0.071	0.000125

Nematode speces[1]	Species code	Fedding habits[2]	Prevalence (%)[3]	Nematode abundance			Biomass[4]	SCBD[5]
				Mean	Min	Max		
57. *Merlinius brevidens*	S116	migratory ectoparasite	72.6	35.8	2	176	0.185	0.039512
58. *Nagelus obscurus*	S118	migratory ectoparasite	0.3	28	28	28	0.137	0.000178
59. *Neodolichorhynchus microphasmis*	S122	migratory ectoparasite	0.3	42	42	42	0.460	0.001402
60. *Neopsilenchus* sp.	S124	microherviborous	0.3	31	31	31	0.188	0.000211
61. *Ogma civellae*	S126	migratory ectoparasite	1.1	13.2	3	38	1.033	0.003519
62. *Ogma palmatum*	S128	migratory ectoparasite	0.3	6	6	6	1.175	0.000424
63. *Ogma rhombosquamatum*	S129	migratory ectoparasite	20.5	483.3	1	9800	0.569	0.018593
64. *Paratrichodorus "allius"*	S131	migratory ectoparasite	2.7	5.2	1	12	1.324	0.001412
65. *Paratrichodorus* sp 1	S134	migratory ectoparasite	0.8	9.7	3	14	0.291	0.000656
66. *Paratrichodorus* sp 10	S135	migratory ectoparasite	0.3	4	4	4	0.645	0.000039
67. *Paratrichodorus* sp 15	S138	migratory ectoparasite	0.3	18	18	18	0.685	0.000096
68. *Paratrichodorus* sp 3	S140	migratory ectoparasite	0.3	3	3	3	0.633	0.000149
69. *Paratrichodorus* sp 4	S141	migratory ectoparasite	0.8	1.7	1	3	0.622	0.000096
70. *Paratrichodorus* sp 6	S143	migratory ectoparasite	0.3	15	15	15	0.698	0.000249
71. *Paratrichodorus* sp 9	S145	migratory ectoparasite	0.3	3	3	3	0.614	0.000010
72. *Paratrophurus loofi*	S147	migratory ectoparasite	1.9	14.9	3	56	0.748	0.004933
73. *Paratrophurus striatus*	S148	migratory ectoparasite	0.3	12	12	12	0.295	0.000156
74. *Paratylenchus ciccaronei*	S150	migratory ectoparasite	6.6	132.2	3	974	0.047	0.002823
75. *Paratylenchus microdorus*	S151	migratory ectoparasite	23.1	185.1	2	7480	0.074	0.014765

Nematode speces[1]	Species code	Fedding habits[2]	Prevalence (%)[3]	Nematode abundance			Biomass[4]	SCBD[5]
				Mean	Min	Max		
76. *Paratylenchus sheri*	S152	migratory ectoparasite	8.5	347.4	4	3024	0.109	0.019067
77. *Paratylenchus vandenbrandei*	S153	migratory ectoparasite	13.6	137.7	4	1736	0.027	0.013655
78. *Pratylenchoides alkani*	S154	migratory endoparasite	0.8	29	2	49	0.594	0.000389
79. *Pratylenchoides crenicauda*	S155	migratory endoparasite	0.3	38	38	38	0.310	0.000119
80. *Pratylenchoides hispaniensis*	S156	migratory endoparasite	0.3	2	2	2	0.482	0.000006
81. *Pratylenchoides ritteri*	S157	migratory endoparasite	0.5	68.5	48	89	0.408	0.001098
82. *Pratylenchus neglectus* [6]	S159	migratory endoparasite	6.4	10.8	2	47	0.097	0.000717
83. *Pratylenchus oleae*	S160	migratory endoparasite	0.8	55	28	98	0.099	0.002373
84. *Pratylenchus penetrans*	S161	migratory endoparasite	0.8	14.3	7	19	0.171	0.000496
85. *Pratylenchus scribneri*	S162	migratory endoparasite	0.3	24	24	24	0.139	0.000186
86. *Pratylenchus thornei* [6]	S169	migratory endoparasite	17	16.8	1	126	0.148	0.007639
87. *Psilenchus hilarulus*	S172	microherviborous	7.4	11.1	1	121	0.391	0.005227
88. *Psilenchus hilarus*	S173	microherviborous	0.8	6	2	7	0.388	0.000259
89. *Rotylenchulus macrosoma**	S176	semiendoparasite	0.5	508.5	141	876	0.048	0.000528
90. *Rotylenchus cypriensis*	S177	migratory ectoparasite	0.3	57	57	57	0.185	0.000407
91. *Rotylenchus incultus*	S180	migratory ectoparasite	3.5	277.5	4	1230	0.317	0.012569
92. *Rotylenchus* sp 1	S184	migratory ectoparasite	0.5	31	3	59	0.918	0.000875
93. *Rotylenchus* sp 5	S188	migratory ectoparasite	0.3	8	8	8	0.942	0.000029
94. *Trichodorus andalusicus*	S191	migratory ectoparasite	8.5	16.3	1	57	0.190	0.002358

Nematode speces[1]	Species code	Fedding habits[2]	Prevalence (%)[3]	Nematode abundance			Biomass[4]	SCBD[5]
				Mean	Min	Max		
95. *Trichodorus giennensis*	S192	migratory ectoparasite	7.7	10.9	1	58	0.235	0.002674
96. *Trichodorus onubensis*	S195	migratory ectoparasite	0.3	6	6	6	0.518	0.000339
97. *Trichodorus paragiennensis*	S196	migratory ectoparasite	0.5	11	3	19	0.497	0.000096
98. *Trichodorus parasilvestris*	S197	migratory ectoparasite	0.3	34	34	34	0.367	0.000134
99. *Trichodorus* sp. AMS-2014	S199	migratory ectoparasite	0.3	12	12	12	1.226	0.000050
100. *Trophurus imperialis*	S201	migratory ectoparasite	0.8	11	1	23	0.558	0.000891
101. *Tylenchorhynchus clarus*	S204	migratory ectoparasite	11.1	121.7	4	2072	0.108	0.014698
102. *Tylenchorhynchus laeviterminalis*	S207	migratory ectoparasite	0.8	89	10	178	0.159	0.000703
103. *Tylenchorhynchus mediterraneus*	S209	migratory ectoparasite	7.2	91.6	3	672	0.511	0.025731
104. *Tylenchorhynchus zeae*	S205	migratory ectoparasite	0.5	19.5	12	27	0.153	0.002521
105. *Tylenchus davainei*	S215	microherviborous	21.5	27	2	252	0.594	0.031589
106. *Tylenchus elegans*	S216	microherviborous	30.8	19.8	2	127	0.447	0.025444
107. *Tylenchus magnus*	S218	microherviborous	0.5	10.5	4	17	0.481	0.000132
108. *Tylenchus* sp.	S220	microherviborous	0.3	3	3	3	0.517	0.000231
109. *Xiphinema adenohystherum*	S222	migratory ectoparasite	0.8	1.7	1	2	11.346	0.001251
110. *Xiphinema baetica*	S225	migratory ectoparasite	0.3	1	1	1	9.897	0.000106
111. *Xiphinema cadavalense*	S226	migratory ectoparasite	0.5	1	1	1	20.547	0.000989
112. *Xiphinema coxi europaeum*	S230	migratory ectoparasite	1.3	6.2	1	18	7.494	0.004608
113. *Xiphinema duriense*	S231	migratory ectoparasite	0.3	1	1	1	0.666	0.000129

Nematode speces[1]	Species code	Fedding habits[2]	Prevalence (%)[3]	Nematode abundance Mean	Min	Max	Biomass[4]	SCBD[5]
114. *Xiphinema hispidum*	S234	migratory ectoparasite	0.3	30	30	30	4.040	0.001856
115. *Xiphinema incertum*	S235	migratory ectoparasite	0.3	38	38	38	1.319	0.002035
116. *Xiphinema index*	S236	migratory ectoparasite	0.3	3	3	3	5.426	0.000946
117. *Xiphinema italiae*	S237	migratory ectoparasite	8.5	11.6	1	59	1.882	0.014404
118. *Xiphinema iznajarense*	S262	migratory ectoparasite	0.3	34	34	34	11.564	0.003091
119. *Xiphinema macrodora*	S239	migratory ectoparasite	0.5	11	8	14	51.391	0.004781
120. *Xiphinema mengibarense*	S263	migratory ectoparasite	0.3	21	21	21	6.359	0.001333
121. *Xiphinema nuragicum*	S241	migratory ectoparasite	9.3	21.9	1	218	9.295	0.040846
122. *Xiphinema pachtaicum*	S244	migratory ectoparasite	70.4	35.7	1	819	1.047	0.091476
123. *Xiphinema* sp 4	S253	migratory ectoparasite	0.3	1	1	1	4.308	0.001123
124. *Xiphinema* sp 5	S171	migratory ectoparasite	0.5	20	12	28	0.841	0.004496
125. *Xiphinema turcicum*	S255	migratory ectoparasite	1.3	9.6	2	22	8.885	0.007783
126. *Xiphinema turdetanense*	S256	migratory ectoparasite	0.5	2.5	1	4	8.792	0.000213
127. *Xiphinema vallense*	S257	migratory ectoparasite	0.5	14	12	16	1.084	0.000623
128. *Zygotylenchus guevarai* [6]	S258	migratory endoparasite	6.9	26.6	2	264	0.100	0.002409

[1] For species identification see: (Vovlas *et al.* 2008, Castillo *et al.* 2010, Gutiérrez-Gutiérrez *et al.* 2010, 2012, 2013a, 2013b, Palomares-Rius *et al.* 2010, 2014, 2017a, 2017b Cantalapiedra-Navarrete *et al.* 2013, Decraemer *et al.* 2013, Handoo *et al.* 2014, Subbotin *et al.* 2014, 2015, Van den Berg *et al.* 2014, Archidona-Yuste *et al.* 2016a, b, c, d, 2018, Van Den Berg *et al.* 2016)

[2] Feeding habits according to Yeates et al. (1993).

[3] Prevalence was calculated as the percentage of samples in which a nematode species was diagnosed with respect to total number of samples.

[4] Relative nematode wet biomass according to an adjusted Andrassy´s formula (Andrássy 1956); relative biomass (µg) = L x D^2 / 1.600.000; where L is nematode body length (in µm); and D is nematode maximum body width (in µm). (*) Biomass based on second-stage juveniles.

[5] SCBD: species contribution to beta diversity (Legendre and De Cáceres 2013).

[6] Plant-parasitic nematodes species could be associated with cultivated and wild legumes growing as cover crops rather than with cultivated olives; as olive is not a suitable host for this PPN species (Nico 2003, Davis and Venette 2004, Castillo and Vovlas 2007, Castillo *et al.* 2010)

2. Experimental Design, Materials, and Methods

2.1 Study area

The cultivated olive growing of southern Spain, Andalusia was selected as study area. A total of 376 commercial olive orchards were selected across the entire olive area of Andalusia (Archidona-Yuste *et al.* 2018) (Table 2.1). The systematic survey covered the diversity of olive cropping systems including agroforestry stands, traditional groves and new intensive orchards (Palomares-Rius *et al.* 2015, CAP-JA 2016, Archidona-Yuste *et al.* 2018).

Approximately 50% of the total surface area of Andalusia region is covered with natural and forest vegetation, and 44% by agricultural areas which are associated with olive orchards, cereal crops and vineyards (Bermejo *et al.* 2011). Although cultivated olive is extensively grown in the Mediterranean Basin, in Andalusia this cultivated non-tropical fruit tree covers more than 1.6 million ha accounting for 19% of the total region surface area in an impressive monoculture (CAP-JA 2016, MAGRAMA 2016). In fact, historically and to present times cultivated olive has an essential contribution to industrialization of agriculture, being culturally and economically very relevant for the Mediterranean area, especially for Andalusia (Infante-Amate 2012).

2.2 Sampling design

Data was obtained by systematic survey based on sampling design described by Archidona-Yuste *et al*. (2018). In brief, soil samples were collected from 2011 to 2016 during the spring season. In each commercial olive orchard, soil samples were taken from four to five looking healthy trees that were georeferenced. Soil samples were collected with a hoe discarding the upper 5-cm top soil profile, from a 5- to 50-cm depth, in the close vicinity of active olive roots. In fact, we ensured that roots from other plants including weeds or other herbaceous plants were not included. Finally, all individual samples were thoroughly mixed to obtain a single representative sample per each commercial olive orchard before nematode

extraction and physicochemical parameters determination (Archidona-Yuste *et al.* 2018).

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Table 2.3 Explanatory variables sets used to assess community composition and species richness[1].

Variable[1]	Description	Source data[2]
Environment		
Bioclimatic predictors		
BIO1	Annual Mean Temperature (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO2	Annual Mean Diurnal Range (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO3	Isothermality (%)	O´Donnell and Ignizio (2012); REDIAM
BIO4	Temperature Seasonality (Standard Deviation) (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO5	Max Temperature of Warmest Month (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO6	Min Temperature of Coldest Month (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO7	Annual Temperature Range (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO8	Mean Temperature of Wettest Quarter (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO9	Mean Temperature of Driest Quarter (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO10	Mean Temperature of Warmest Quarter (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO11	Mean Temperature of Coldest Quarter (ºC)	O´Donnell and Ignizio (2012); REDIAM
BIO12	Annual Precipitation (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO13	Precipitation of Wettest Month (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO14	Precipitation of Driest Month (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO15	Precipitation Seasonality (CV) (%)	O´Donnell and Ignizio (2012); REDIAM

Variable[1]	Description	Source data[2]
BIO16	Precipitation of Wettest Quarter (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO17	Precipitation of Driest Quarter (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO18	Precipitation of Warmest Quarter (mm)	O´Donnell and Ignizio (2012); REDIAM
BIO19	Precipitation of Coldest Quarter (mm)	O´Donnell and Ignizio (2012); REDIAM
Topographic		
Aspect	Average aspect of olive orchard computed by DEM of 5 m scale resolution	NGI
Slope	Average slope of olive orchard computed by DEM of 5 m scale resolution	NGI
SWI	Saga Wetness Index describes how susceptible specific areas are to becoming saturated if high inputs of precipitation occur in a relatively short amount of time.	SAGA
GR	Annual mean Global Solar Radiation on olive orchard for 2010	REDIAM
Other predictors		
PET	Annual mean Olive Potential Evapotranspiration for the period 2009-2015 (mm)	REDIAM
DI	Annual mean Standardized Drought Index is the standardization of cumulative monthly rainfall anomalies for the period 2011-2015	REDIAM
RD	Mean Annual Intensity of Rainfall Deficit is based on the calculation of rainfall anomalies for the period 2005-2015 regarding to the mean precipitation of the historical reference series 1971-2000	REDIAM
CA	Climate Areas represent homogeneous areas according to precipitation regimen and temperature (see Table A3)	REDIAM
Soil		
Soil Chemistry		
CEC	Soil Cation Exchange Capacity (meq/100g)	Data collection

Variable[1]	Description	Source data[2]
Ca	Soil Calcium content (meq/100g)	Data collection
Mg	Soil Magnesium content (meq/100g)	Data collection
Na	Soil Sodium content (meq/100g)	Data collection
Kech	Soil exchangeable Potassium content (meq/100g)	Data collection
CO3	Soil Carbonate content (%)	Data collection
Pext	Soil extractable Phosphorus content (ppm)	Data collection
SOM	Soil Organic Matter content (%)	Data collection
Corg	Total Soil Carbon content (%)	Data collection
Norg	Total Soil Nitrogen content (%)	Data collection
C:N	Soil Carbon/Nitrogen ratio	Data collection
pH (KCl)	Soil pH determination in KCl solution	Data collection
Soil Texture		
Clay	Soil Clay content (%)	Data collection
Sand	Soil Sand content (%)	Data collection
Silt	Soil Silt content (%)	Data collection
Soil edaphic unit	Any kind of soils presented in olive orchard (see Table A4)	REDIAM
Agronomic management		
Cultivar	Olive tree cultivar growing on commercial olive orchard (see Table A5)	Data collection
Age	Age of olive orchard (years)	Data collection

Variable[1]	Description	Source data[2]
Density	Proportion of olive trees per ha on olive orchard (traditional, intensive and super high-density olive orchards)	Data collection
Irrigation	Irrigation regimen in olive orchard (e.g. rainfed or irrigated)	Data collection
Irrigation system	Irrigation system installed on olive orchard (e.g. drip or blanket irrigation system)	Data collection
Water	Source of irrigation water (e.g. underground or superficial water source)	Data collection
Canopy	Agronomic management practices below olive tree canopy on olive orchard (e.g. herbicide application / tilling or not management practices)	Data collection
Alley	Agronomic management practices on alley of olive orchard (e.g. tilling, herbicide application, grinding or vegetative cover)	Data collection
Alley Cover	Any kind of vegetative cover on alley of olive orchard (e.g. natural or grasses vegetation)	Data collection

[1] Variables regarding to data collection were measured at the scale of the olive orchard.

[2] Source data: Bioclimatic predictors computed according to O´Donnell and Ignizio (2012) with data from REDIAM (Environmental Information Network of Andalusia; http://www.juntadeandalucia.es/medioambiente/site/rediam) (REDIAM 2016); NGI (National Geographic Institute of Spain; http://www.ign.es/ign/main/index.do?locale=en); SAGA (calculated with module SAGA Wetness Index (Bohener *et al.* 2002)

2.3 Nematode extraction

From each soil sample, nematodes were extracted separately from two 250-cm^3 subsamples using magnesium sulphate centrifugal-flotation method (Coolen 1979, Castillo *et al.* 2010). Soil was washed thoroughly with tap water through a 710-µm mesh sieve, and the filtered water was collected in a beaker and extensively mixed with 4% kaolin (v/v). This mixture was centrifuged at 1,100 x g for 4 min, and the supernatants discarded. Pellets were re-suspended in 250 ml MgSO4 (δ = 1.16) and the new suspensions were centrifuged at 1,100 x g for 3 min. The supernatants were sieve through a 5 µm mesh, and nematodes collected on the sieve were washed with tap water (Coolen 1979). Water solution containing nematodes collected from each of the two 250 cm^3 were mixed in a single one in order to carry out the diagnostic and identification of nematodes from a 500 cm^3 soil subsample.

Table 2.4 Phytoclimatic types areas presented in commercial olive orchards sampled.

Phytoclimatic types	Code	Commercial olive orchards
Sub-Mediterranean	1	16
Warm semi-arid Mediterranean	2	57
Dry warm Mediterranean	3	72
Warm less dry Mediterranean	4	204
Mediterranean moderate	5	6
Sub-humid Atlantic Mediterranean	6	20
Central European Mediterranean	7	1
Total number		376

Note: Phytoclimatic type areas were obtained from the Environmental Information Network of Andalusia (REDIAM 2016)

2.4 Nematode identification

In order to select the PPN from the global nematode community in the soil, the nematode sample was poured into a counting dish (8 cm L x 8 cm W x 1.5 cm H), where they were identified and then, counted under a stereo-

microscope (Leica MZ12; Leica Microsystems, Wetzler, Germany). PPN were identified to genus, and then we focused on the species delineation selecting adult nematode specimens which were fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerine using Seinhorst´s method (Seinhorst 1962), and identified by morphological traits and molecular markers to species level. The morphological study at nematode species level was performed by classical diagnostic features using general and specific taxonomic keys from each nematode family and genus. However, the identification of nematode species based solely on morphological diagnostic is quite complex due to the occurrence of cryptic species and/or overlapping of morphological diagnostic characters among PPN species (Cantalapiedra-Navarrete *et al.* 2013, Palomares-Rius *et al.* 2015, Archidona-Yuste *et al.* 2016a). Therefore, polyphasic identification, based on an integrative taxonomy of combining both molecular and morphological techniques, was performed to get an efficient and reliable identification of PPN species (*see Notes* in Table 2.2).

2.5 Prevalence, abundance, biomass, and species richness calculation

Prevalence was calculated by dividing the number of samples in which PPN species was detected by the total number of samples and expressed as a percentage. Total nematode abundance in each commercial orchard was calculated as the total number of specimens from all species identified per 500 cm^3 of soil for each commercial olive orchard. For each species identified, the abundance was calculated as the total number of specimens per 500 cm^3 of soil. Relative nematode individual fresh biomass was calculated according to an adjusted Andrassy´s formula (Andrássy 1956), wherein relative biomass (µg) = $L \times D^2 * 1{,}600{,}000^{-1}$; where L is nematode body length (in µm), and D is nematode maximum body width (in µm). Nematode size was determined with indications described by Archidona- Yuste et al., (Archidona-Yuste *et al.* 2018). In addition, nematode species richness was determined for each olive orchard.

Table 2.5 Soil types presented in commercial olive orchards sampled.

Soil edaphic unit[1]	Code	Commercial olive orchards[2]	Soil edaphic unit[1]	Code	Commercial olive orchards[2]
Bc Re I U	31	10	Lc Re I H Be	54	1
Bc U Lo Lc	33	1	Lk Bk Be Lc Rc I	57	10
Be Lc Bk Rc Lk	36	1	Lk Bk Lc Rc	58	31
Be Lc Lo	37	13	Lk Lc Lg	59	5
Be Re Lc I	32	7	Lo Lg Be	51	3
Bk Bg Rc	45	1	Lv Lg	50	1
Bk Lk Lc Jc	47	17	Qa Bh Gd	20	1
Bk Rc	41	1	Rc Bk I Jc	13	72
Bk Rc I E	44	30	Rc Bk Lk Jc	14	10
Bk Rc I Jc Bv	43	11	Rc I Bk	11	9
Bk Rc Jc Lc	42	10	Re Be Lo I	8	2
Bv Rc Vc Bk	48	12	Re I Ce	5	13
Bv Vc Bk Rc	49	8	Vc Be Bk Rc Vp	23	18
Fe Ce	1	1	Vp E Rc	21	2
I Bk Xk	18	2	Vp Vc	22	4
I Lc E Bk	19	10	We Lg Lp	61	9
I Re Lc Be	15	1	Xk I Jc	29	1
Jc	2	25	Xk Rc jc	28	2
Jc Xk Rc	3	1	Xk Xl Rc Jc	30	9
Lc Be I	56	3			
Lc Bk I	52	5	Total number		376
Lc I Re Nd	55	2			

[1] Soil edaphic unit according to FAO (FAO 1974).

[2] Number of commercial olive orchards sampled from the total of 376 with each soil edaphic units.

2.6 Explanatory variables data sets

2.6.1 Environmental variable data set

All explanatory variables were recorded as continuous data (except of categorical CA) in raster layers from which individual values for each olive orchard were extracted using the raster to point tool in QGIS (QGIS Development Team 2016). The topographic variables for a given commercial olive orchard were the mean of the values derived for each olive tree (from four to five). Temperature and precipitation data were obtained from the Environmental Information Network of Andalusia (REDIAM) for the period 1970-2012 at 100 m ground resolution (REDIAM 2016), from which 19 bioclimatic variables were derived according to procedures described by (O´Donnell and Ignizio 2012). These variables were calculated using the R package *dismo* (Hijmans *et al.* 2016). GIS-derived topographic variables included the continuous variables slope, aspect and SWI. Each of these variables was derived in QGIS using a digital elevation model (DEM) obtained from the Spanish National Geographic Institute at a 5 m ground resolution (NGI 2016). Slope and aspect variables were calculated from DEM according to second-degree polynomial adjustment method (Zevenbergen and Thorne 1987) using the library morphometry of the open source GIS SAGA (Conrad *et al.* 2015). Because of the soil nematodes biological activity is influenced by the soil moisture (Norton 1978), spatial soil moisture patterns caused by topography variability could influence the distribution of PPN community composition as in other soil processes or properties resulting in vegetation patterns (Zinko *et al.* 2005). SWI which predicts potential areas with relative higher soil moisture, was computed from elevation DEM using the hydrology module in SAGA (Boehner and Selige 2006). Slope ranged with values from 0.08 to 35.5º, and aspect, defined as the direction to which a slope faces, ranged from 0.06 to 359.9º. Since aspect is a circular variable measured in degrees, it was transformed into a categorical variable with six categories (i.e. northeast, east, southeast, southwest, west, and northwest) (Legendre *et al.* 2009).

Table 2.6 Soil texture classification from commercial olive orchards in Andalusia (southern Spain).

Soil Texture Code[1]	Name	Commercial Olive Orchards[2]
Cl	clay	41
SiCl	silty clay	30
SaCl	sandy clay	0
ClLo	clay loam	74
SiClLo	silty clay loam	38
SaClLo	sandy clay loam	26
Lo	loam	76
SiLo	silty loam	13
SaLo	sandy loam	64
Si	silt	0
LoSa	loamy sand	12
Sa	sand	2
	Total number	376

[1] Soil texture classes according to USDA soil texture classification (USDA 1993).
[2] Number of commercial olive orchards sampled from the total of 376 with each soil texture classes.

2.6.2 Soil variables data set

In order to avoid collinearity among texture variables, soil texture from the 376 commercial olive orchards sampled was categorized in 12 texture classes according to the USDA soil texture classification (USDA 1993) (Table 2.6). This analysis was performed using the package soiltexture using the R software (Moeys 2015). Soil texture was estimated by the relative amounts of sand, clay and silt according to soil texture Bouyoucos method (FAO 1980), which values ranging 1.3-90.5%, 3.5-64.1% and 3.7-71.3%, respectively.

Table 2.7 Commercial olive orchards number distributed following agronomic management studied (- = not determined).

	Irrigation		Alley agronomic management				Canopy agronomic management		Density			
Olive cultivar	rainfed	irrigated	vegetative cover	grinding	herbicide	tilling	Tilling/ herbicide	nothing	traditional	intensive	superintensive	Total number
Aloreña	1	0	1	0	0	0	1	0	0	1	0	1
Arbequina	7	32	10	2	15	12	11	28	-	10	-	39
Cornicabra	1	1	0	0	0	2	2	0	-	-	-	2
Frantoio	0	1	0	0	0	1	0	1	-	-	-	1
Gordal	4	1	2	1	1	1	1	4	1	1	-	5
Hojiblanca	14	14	7	1	11	9	11	17	4	3	-	28
Koroneiki	0	2	0	0	2	0	0	2	-	-	-	2
Lechín	1	2	1	0	1	1	2	1	1	-	-	3
Granada Lechín	24	6	15	0	3	12	21	9	11	4	-	30
Manzanilla Serrana	9	1	9	0	0	1	10	0	9	-	-	10
Manzanilla Sevilla	15	5	7	2	4	7	13	7	5	2	-	20
Nevadillo	4	0	3	0	0	1	4	0	3	-	-	4
Pico	1	0	1	0	0	0	1	0	1	-	-	1
Limón Picual	124	74	91	6	43	58	104	94	85	16	2	198
Picudo	4	6	5	0	4	1	5	5	4	1	-	10
Shikitita	0	1	1	0	0	0	0	1	0	0	1	1
Verdial	8	2	8	0	2	0	7	3	8	-	-	10
Mixture of various	11	0	6	0	4	1	8	3	6	-	-	11
Total number	230	146	167	12	91	107	349	27	204	38	3	376

2.6.3 Agronomic management variables data set

Data on plant density was categorized into three classes (e.g. traditional, intensive and super high-density olive orchards) according to Rallo et al. (Rallo *et al.* 2013). The age of olive orchard was determined according to information provided by the landowner, and ranged from about 2 to 100 years that belonged to 21 olive cultivars.

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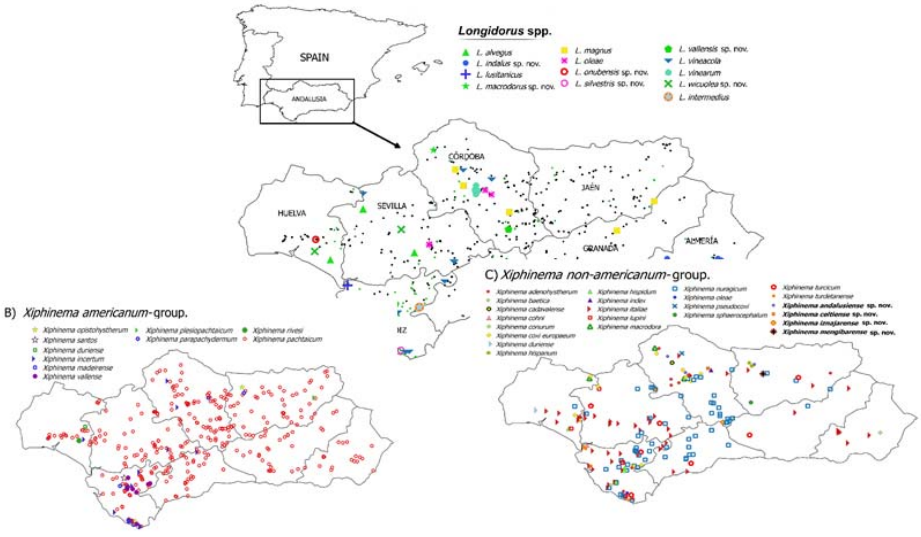
3 BLOQUE II

LA FAMILIA LONGIDORIDAE EN OLIVO EN ANDALUCIA



C2

Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematode: Longidoridae) as inferred from morphometrics and molecular markers

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Abstract

The *Xiphinema americanum*-group constitutes a complex of about 55 species of polyphagous plant-ectoparasitic nematodes with a worldwide distribution. This group of plant-parasitic nematodes is one of the most difficult dagger nematode species complexes for diagnosis because the morphology is very conservative and morphometric characters often overlap. We conducted nematode surveys in cultivated and wild olives in southern Spain from 2012 to 2014, from which we identified 16 nematode populations of the *X. americanum*-group, five of which were tentatively identified as belonging to three new species and are described herein as ***Xiphinema plesiopachtaicum* sp. nov., *Xiphinema vallense* sp. nov**., and ***Xiphinema astaregiense* sp. nov.**, and 11 populations belonging to nine known species: *Xiphinema brevisicum*, *Xiphinema duriense*, *Xiphinema incertum*, *Xiphinema luci*, *Xiphinema madeirense*, *Xiphinema opisthohysterum*, *Xiphinema pachtaicum*, *Xiphinema parapachydermum*, and *Xiphinema rivesi*. A phenetic study based on multivariate factor analyses was developed to compare some of these related species by using morphometric features. In the factor analysis the first four factors accounted for 73.1% of the total variance of the selected characters, identifying body length, body length/maximum body width (a), body length/pharyngeal length (b), body length/tail length (c), and tail length/body width at anus (c′) ratios, distance from anterior end to vulva as percentage of body length (V), stylet length, oral aperture-guiding ring distance, and lip region width as key morphometric characters to differentiate a restricted set of species within the *X. pachtaicum*-subgroup that includes ***X. plesiopachtaicum* sp. nov**. and ***X. vallense* sp. nov**. Multivariate analysis of variance using these specific characters allowed to differentiate species in the *X. pachtaicum* complex or groups of them using morphometric characters (body length, a, b, c, c′, V, stylet length, lip region width, oral aperture guiding ring distance, female tail length, and hyaline region length). Phylogenetic analyses based on nuclear ribosomal DNA genes [D2-D3 expansion segments of large ribosomal subunit 28S, and internal transcribed spacer 1 (ITS1)] and the protein-coding mitochondrial gene, cytochrome c oxidase subunit 1 (coxI) were congruent, showing two main clades separating most of the species of *X. americanum*-subgroup 'sensu stricto' from the *X. pachtaicum*-subgroup. Agreement between phylogenetic

trees and some morphological characters (*viz.* total stylet length, vulva position, and a ratio) were tested by reconstruction of their histories on rRNA-based trees using parsimony and Bayesian approaches. Thus, integrative taxonomy, based on a combination of multivariate morphological and molecular analyses constitutes a new insight into the identification of *X. americanum*-group species.

ADDITIONAL KEYWORDS: Bayesian inference – cryptic species – cytochrome c oxidase subunit 1 (coxI) – D2-D3 – expansion segments of large ribosomal subunit (28S) – dagger nematodes – factor analysis – internal transcriber spacer – MANOVA – rRNA.

1. Introduction

Dagger nematodes of the genus *Xiphinema* comprise plant-parasitic species that damage a wide range of wild and cultivated plants through direct feeding on root cells or transmission of plant pathogenic viruses (Taylor and Brown 1997). Owing to its large morphological diversity, the genus *Xiphinema* was divided into two different species groups (Loof and Luc 1990, Lamberti *et al.* 2000, Coomans *et al.* 2001): (1) the *Xiphinema americanum*-group, which comprises a complex of about 55 species, many of them with a cosmopolitan distribution, and characterized by a spiral or C-shaped medium to small body, postequatorial vulva position, female reproductive system with two equally developed genital branches, usually with short uteri without uterine differentiation, and short conical to broadly convex-conoid tail; and (2) the *Xiphinema* non-*americanum*- group, which comprises a complex of more than 200 species, characterized by a longer body and odontostyle, usually with long uteri and uterine differentiation (including the 'Z-organ', spines, or crystalloid structures in the tubular part of the uterus). Some species of both groups are vectors of several important plant viruses that cause significant damage to a wide range of crops. This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* spp. vectors. Only nine of the approximately 260 known species of *Xiphinema* have been shown able to transmit nepoviruses (genus *Nepovirus*, family *Comoviridae*; (Decraemer and Robbins 2007).

Species of the *X. americanum*-group include the vectors of a wide range of crops, including *Tobacco ringspot virus* (*TRSV*), *Tomato ringspot virus* (*TomRSV*), *Cherry rasp leaf virus* (*CRLV*), and *Peach rosette mosaic virus* (*PRMV*) (Taylor and Brown 1997). The high number of species within the *X. americanum*-group and their well-conserved and overlapping morphometric features complicate their identification. Therefore, complex polytomous and dichotomous keys, based on a combination of major diagnostic characters, have been constructed to enable morphological identification (Lamberti *et al.* 2000, Lamberti *et al.* 2004). Although several polytomous keys have been published (Lamberti et al. 2000, 2004), ambiguities still persist because of poorly defined species boundaries and overlapping morphometrics (Luc and Baujard 2001). Thus, species

differentiation remains difficult and species diversity and taxonomic validity of species are controversial, with the number of recognized species ranging from 34 (Luc *et al.* 1998, Coomans *et al.* 2001) to more than 50 (Lamberti *et al.* 2000, Gozel *et al.* 2006, Gutiérrez-Gutiérrez *et al.* 2012). Accurate identification of *X. americanum*-group species is essential because several species of this group are listed as A1 (*Xiphinema americanum* Cobb, 1913, *Xiphinema californicum* Lamberti and Bleve-Zacheo, 1979, *Xiphinema bricolense* Ebsary, Vrain and Graham, 1989) and A2 (*Xiphinema rivesi* Dalmasso, 1969) quarantine organisms by the European and Mediterranean Plant Protection Organisation (http://www.eppo.int/).

There is controversy concerning the nature of the 'true' *X. americanum*-group i.e. only grouping those species that show the presence of symbiotic bacteria with males absent or rare. Loof and Luc (1990) excluded *Xiphinema pachydermum* Sturhan, 1983, from the *X. americanum*-group because the uteri are long, the oocytes lack bacteria, and it is a bisexual species with numerous males. This action by Loof and Luc (1990) was confirmed by the morphological phylogeny carried out by Coomans et al. (2001), in contradiction to Lamberti and Ciancio (1993, 1994) and Lamberti et al. (2000) who considered the *X. americanum*-species group in a broader sense and included *X. pachydermum* within the *X. pachtaicum*-subgroup comprised of seven species (*Xiphinema fortuitum* Roca, Lamberti and Agostinelli, 1987 (Roca *et al.* 1987) , *Xiphinema incertum* Lamberti, Choleva and Agostinelli, 1983 (Lamberti *et al.* 1983), *Xiphinema madeirense* Brown et al. 1992 (Brown *et al.* 1992), *X. pachydermum*, *Xiphinema simile* Lamberti, Choleva and Agostinelli, 1983, *Xiphinema utahense* Lamberti and Bleve-Zacheo, 1979 (Lamberti and Bleve-Zacheo 1979), and *Xiphinema opisthohysterum* Siddiqi, 1961.

Species determination of the *X. americanum*-group is complex, difficult, and time-consuming. The application of molecular methods in order to determine nematode population structure and systematics has revealed that some long-assumed single species are in fact cryptic species that are morphologically indistinguishable but may be phylogenetically distant to one other (Ye *et al.* 2004, Oliveira *et al.* 2005, 2006, Wu *et al.* 2007, Barsi and Luca 2008, Gutiérrez-Gutiérrez *et al.* 2010, 2012). During the last decade, sequences of nuclear ribosomal DNA (rDNA) and mitochondrial DNA

(mtDNA) have been used for molecular characterization and reconstruction of phylogenetic relationships within the Longidoridae and more particularly within *Xiphinema* (Ye *et al.* 2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2011b, 2012, Subbotin *et al.* 2014). Several genes have been used for molecular characterization of these nematode groups and have provided useful tools for species differentiation, including the partial 28S ribosomal ribonucleic acid (rRNA) gene, internal transcribed spacer (ITS) rRNA gene, 18S rRNA gene, and the mitochondrial genes cytochrome c oxidase subunit 1 (coxI) and nicotinamide dehydrogenase subunit 4 (nad4) (Ye *et al.* 2004, He *et al.* 2005, Kumari *et al.* 2010, Gutiérrez-Gutiérrez *et al.* 2011a, 2011b, 2012, Kumari and Subbotin 2012, Subbotin *et al.* 2014, Zasada *et al.* 2014). Sequence data from the 28S D2–D3 expansion region of the rRNA revealed two well-supported clades of the *X. americanum*-group. However, this marker did not differentiate some of the species, whereas the ITS region was more informative for molecular confirmation (Gutiérrez-Gutiérrez *et al.* 2012, Zasada *et al.* 2014).

Consequently, currently available molecular techniques may help to provide tools for differentiating *X. americanum*-group species that could significantly improve and facilitate the routine identification of these nematodes. Polyphasic identification, based on an integrative strategy of combining molecular techniques with morphology and measurements for species diagnosis, has proved to be an efficient and reliable tool for nematode identification within this and other nematode groups (Derycke *et al.* 2010, Meza *et al.* 2011, Gutiérrez-Gutiérrez *et al.* 2012, 2013). In Spain, the *X. americanum*-group was recently studied by Gutiérrez-Gutiérrez *et al*. (2010, 2012) on populations from grapevines and other host-plants in southern Spain, where two new species were described (*Xiphinema parapachydermum* and *Xiphinema paratenuicutis*), and four new records for the country were reported (*Xiphinema duriense*, *X. incertum*, *X. opisthohysterum*, *Xiphinema santos*). In that research, species delineation of the *X. americanum*-group was based upon the integrative application of morphological, morphometric, and molecular methods in order to unravel potential cryptic species diversity. Morphological and morphometric studies as well as molecular sequencing were used simultaneously to group specimens into species, then a multivariate analysis was carried out using

morphometric characters in which a high number of measured individuals was analysed in order to find morphometric differences amongst them.

Olive trees serve as hosts to a large number of plant-parasitic nematodes, of which root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.), spiral nematodes (*Helicotylenchus* spp.), and dagger nematodes (*Xiphinema* spp.) are widely distributed and damage this crop (Castillo *et al.* 2010, Ali *et al.* 2014). However, little information is available about dagger nematodes associated with cultivated and wild olives. Our intention in the present study was therefore to increase our knowledge of the biodiversity of this nematode group in southern Spain, which was previously studied in grapevine and other natural host plants (Gutiérrez-Gutiérrez *et al.* 2012). We selected several localities in Andalusia, southern Spain, that are representative of locations where both wild and cultivated olives are prevalent in order to study the species diversity of the *X. americanum*-group. The accurate and timely identification of the *X. americanum*-group species infesting cultivated and natural soils is a prerequisite for designing effective management strategies as well as a reliable method allowing distinction between virus vector and nonvirus vector species, and thus assisting in the exclusion of species under quarantine or regulatory strategies. Partial agreement between taxonomy based on morphological characters and molecular markers such as on D2-D3 and partial 18S regions has been observed in species complexes and cryptic species within the *X.* non-*americanum*-group (Oliveira *et al.* 2006, Wu *et al.* 2007, Gutiérrez-Gutiérrez *et al.* 2013). The study by Gutiérrez-Gutiérrez *et al.* (2013) supported the polyphyletic status of some characters, such as the female tail shape and the degree of development of the genital system in species with equally developed genital branches (Gutiérrez-Gutiérrez *et al*. 2013). In addition, these characters appear to have multiple origins, as previously hypothesized in the cladistic analysis of Coomans *et al*. (2001). In a recent study in our group, morphological character evolution demonstrated a feasible ancestral stage for the pseudo-Z-organ and for the absence of spines, and both characters appeared at different points in the evolutionary tree (Gutiérrez-Gutiérrez *et al*. 2013).

The objective of the present study was to test the congruence between morphological and molecular data within the *X. americanum*-group.

Additionally, we aimed to determine (i) how morphologically and morphometrically related the species belonging to the *X. americanum*-species group associated with cultivated and wild olives in southern Spain are in comparison to previous records; (ii) which are the most useful diagnostic morphological and allometric characters for X. pachtaicum-subgroup species; and (iii) how the following: using the D2-D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI gene sequences are how phylogenetically related the *X. americanum*-group populations and species are using the D2-D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI gene sequences.

2. Material and Methods

2.1 Nematode populations and morphological studies

Nematode surveys were conducted from 2012 to 2014 during the spring season in cultivated olive (*Olea europaea* ssp. *europaea*) and wild olive (*Olea europaea* ssp. *silvestris*) in trees southern Spain. Incidental samples were also collected from other host-plants, including grasses (*Lolium* sp.), oak (*Quercus robur* L.), and rose (*Rosa* sp.) (Table 3.1). Samples were collected with a shovel from the upper 50 cm of soil of four to five plants arbitrarily chosen in each locality. Nematodes were extracted from 500 cm^3 of soil by centrifugal flotation (Coolen 1979) and a modification of Cobb's decanting and sieving methods (Flegg 1966). In some cases, additional soil samples were collected afterwards from the same locality in order to obtain sufficient specimens for morphological and/or molecular identification.

Specimens for light microscopy observations were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerine using Seinhorst's method (Seinhorst 1962).

Table 3.1 Taxa sampled for *Xiphinema americanum* group complex species and sequences used in this study.

Species	Sample code	Locality	Host-plant/reference population	Specimens studied morphology/ molecular	D2-D3	ITS1	*coxI*
***Xiphinema astaregiense* sp. nov.**	J174	Jerez de la Frontera (Cádiz, Spain)	grasses	14/1	KP268955	KP268972	KP268977
***Xiphinema plesiopachtaicum* sp. nov.**	AR63	Coto Ríos (Jaén, Spain)	wild olive	32/3	KP268956 KP268957 KP268958	KP268973	-
***Xiphinema vallense* sp. nov.**	AR55	San José del Valle (Cádiz, Spain)	wild olive	23/1	KP268959	KP268974	-
***Xiphinema vallense* sp. nov.**	AR27	Bolonia (Cádiz, Spain)	wild olive	4/1	KP268960	-	-
***Xiphinema vallense* sp. nov.**	H003	Hinojos (Huelva, Spain)	olive	4/1	KP268961	-	-
Xiphinema brevisicum	CARI	Merza (Pontevedra, Spain)	oak	4/1	KP268962	-	KP268978
Xiphinema duriense	ST02	Gibraleón (Huelva, Spain)	olive	3/1	KP268963	-	-
Xiphinema incertum	ST13	Osuna (Seville, Spain)	olive	6/1	KP268964	-	-
Xiphinema luci	IAGQ	Benacazón (Seville, Spain)	rose	3/1	KP268965	KP268975	-
Xiphinema madeirense	AR31	Tarifa (Cádiz, Spain)	wild olive	9/1	KP268966	KP268976	KP268979
Xiphinema opisthohysterum	AR31	Tarifa (Cádiz, Spain)	wild olive	3/1	KP268967	-	-
Xiphinema pachtaicum	ST80	Huevar del Aljarafe (Seville, Spain)	olive	10/1	KP268968	-	-
Xiphinema pachtaicum	AR44	Sorbas (Almeria, Spain)	olive	10/1	*	-	-
Xiphinema parapachydermum	AR62	Bolonia (Cádiz, Spain)	wild olive	8/1	KP268969	-	-
Xiphinema parapachydermum	ST122	Adamuz (Córdoba, Spain)	olive	3/1	KP268970	-	-
Xiphinema rivesi	ST76	Bollullos Par del Condado (Huelva, Spain)	olive	4/1	KP268971	-	-

(-) Not obtained or not performed. (*) Sequenced population but not deposited in GenBank database, since was identical to KP268968

Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1000× magnification. Measurements were carried out using a drawing tube attached to a light microscope, unless otherwise indicated in the text. All measurements were recorded in micrometres (µm). For the line drawings of the new species, light micrographs were imported into CorelDraw software v. X5 and redrawn. All abbreviations used are as defined in Jairajpuri and Ahmad (1992). In addition, a comparative morphological study on the type specimens of one species was conducted with specimens kindly provided by Dr Z.A. Handoo from the USDA Nematode Collection, Beltsville, MD, USA (*viz. Xiphinema madeirense* Brown *et al*. 1992 slides T-4368p–T-4369p).

Nematode populations of *X. americanum*-group species already described were analysed morphologically and molecularly in this study and proposed as standard and reference populations for each species given until topotype material becomes available and molecularly characterized.

2.2 Multivariate analyses

Multivariate analyses were performed to estimate the degree of association between or amongst species and/or specimens within species (Rencher and Christensen 2012). Firstly, a multivariate factor analysis on 16 populations belonging to six species (four known and two new species) of the *X. pachtaicum*-subgroup was performed. Secondly, a multivariate analysis of variance (MANOVA) was used to estimate the relative weight accounted by the morphometric characters used to explain variation due to species, as well as to estimate significant differences between species in pairwise comparisons. Species delineation of nematodes was based on morphology. Multivariate analyses were based upon the morphometric characters used in the polytomous key by Lamberti *et al.* (2004) and Lamberti and Ciancio (1993) that included: L (body length), the ratios a, b, c, c′, V [(distance from anterior end to vulva/body length) × 100], stylet length (odontostyle + odontophore), oral aperture-guiding ring distance, lip region width, female tail length, and hyaline region length. All the nematode populations belonging to the *X. pachtaicum*-subgroup used in the multivariate factor analysis data set were measured and selected based on the availability of molecular data in order to avoid misidentifications in this complex group (Table 3.1; Gutiérrez-

Gutiérrez *et al.* 2011a, b, 2012). Populations of *Xiphinema rivesi* and *Xiphinema luci* were not included because they are morphologically too different, not belonging to the X. pachtaicum-subgroup (Lamberti et al., 2000), and because very few specimens were detected. Analyses were carried out using R v. 3. 1. 1. (R Core Team 2014), http://www.R-project.org/). Prior to analysis, the normality of the data was checked for normality using a test for multivariate variables in the package 'mvnormtest' (Oksanen *et al.* 2017). Factor analysis was performed by a decomposition of the data matrix amongst populations in a Q-mode type analysis using a principal function implemented in the package 'psych' (Revelle 2014). This analysis produced a set of variables (factors) that are linear combinations of the original variables. The new factors are independent of each other and ranked according to the amount of variation accounted for. After the initial extraction by the principal method, an orthogonal varimax raw rotation was used to estimate the factor loadings. Only factors with sum of squares (SS) loadings > 1 were extracted. Additionally, in order to check the species differentiation, quantitative morphometric characters were then subjected to a MANOVA. The canonical option, as well as one degree of freedom contrast for comparisons between the two new species described in this study with those previously described species within the *X. pachtaicum*-subgroup using the general linear model procedure of SAS (Statistical Analysis System v. 9.3; SAS Institute, Cary, NC, USA).

2.3 DNA extraction, PCR, sequencing

For molecular analyses, in order to avoid mistakes in cases of mixed populations in the same sample, two live nematodes from each sample were temporarily mounted in a drop of 1 M NaCl containing glass beads (avoiding nematode crushing) and diagnostic measurements and photomicrographs taken to confirm their identity. These measurements were not used in the morphometric studies or analyses. The slides were then dismantled and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003). One nematode specimen of each sample was transferred to an Eppendorf tube containing 16 µL double-distilled H2O (ddH2O), 2 µL 10× PCR buffer, and 2 µL proteinase K (600 µg mL−1) (Promega, Benelux, The Netherlands) and crushed for 2 min with a microhomogenizer (Vibro Mixer, Zürich, Switzerland). The tubes

were incubated at 65 °C (1 h), then at 95 °C (15 min), and finally at 80 °C (15 min). One microlitre of extracted DNA was transferred to an Eppendorf tube containing: 2.5 µL 10× NH4 (ammoniun) reaction buffer, 0.75 µL MgCl2 (50 mM), 0.25 µL deoxyribonucleotide triphosphate mixture (10 mMeach), 0.75 µL of each primer (10 mM), 0.2 µL BIOTAQ DNAPolymerase (BIOLINE, UK), and ddH2O to a final volume of 25 µL. The D2-D3 expansion segments of 28S rRNA was amplified using the D2A(5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Nunn 1992). The ITS1 region was amplified using the forward primer 18S (5′-TTGATTACGTCCCTGCCCTTT-3′) (Vrain *et al.* 1992) and reverse primer rDNA1 (5′-ACGAGCCGAGTGATCCACCG-3′) (Cherry *et al.* 1997). Finally, the portion of the coxI gene was amplified as described by Lazarova et al. (2006) using the primers COIF (5′-GATTTTTTGGKCATCCWGARG-3′) and COIR (5′-CWACATAATAAGTATCATG-3′).

PCR cycle conditions for the ribosomal DNA markers were: one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, an annealing temperature of 55 °C for 45 s, 72 °C for 3 min, and finally one cycle of 72 °C for 10 min. The cycle for mtDNA was as described by He et al. (2005): 95 °C for 10 min, five cycles at 94 °C for 30 s, 45 °C for 40 s, and 72 °C for 1 min, and a further 35 cycles at 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 1 min, followed by an extension at 72 °C for 10 min. Sequencing of some of the ITS1 rRNA and partial coxI genes of some known *Xiphinema* spp. Identified herein were not successful despite several attempts (Table 3.1), even using different primers (Gutiérrez-Gutiérrez *et al.* 2011b, 2012). PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and used for direct sequencing in both directions using the primers given above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA, USA), using a BigDye Terminator Sequencing Kit v. 3.1 (Applied Biosystems), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under the accession numbers indicated on the phylogenetic trees and in Table 3.1.

2.4 Phylogenetic analysis

D2-D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI sequences of different *X. americanum*-group species from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each data set was chosen according to previously published data (He et al. 2005, Lazarova et al. 2006, Gutiérrez-Gutiérrez et al. 2012). The newly obtained and previously published sequences for each gene were aligned using MAFFT (Katoh *et al.* 2002) with default parameters. Sequence alignments were manually edited using BioEdit (Hall 1999). Percentage similarity between sequences was calculated using the sequence identity matrix using BioEdit. For this, the score for each pair of sequences was compared directly and all gap or place holding characters were treated as a gap; when the position of both sequences has a gap they do not contribute. Phylogenetic analyses of the sequence data sets were performed based on maximum likelihood (ML) using PAUP * 4b10 (Swofford 2002) and Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fitted model of DNA evolution was obtained using jModelTest v. 2.1.7 (Darriba *et al.* 2012) with the Akaike information criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in the phylogenetic analyses. BI analysis under a general time-reversible with invariable sites and a gamma-shaped distribution (GTR + I + G) model for the D2-D3 expansion segment of 28S rRNA, a GTR and gamma-shaped distribution model for the ITS1 region, and a transversional of invariable sites and gamma-shaped distribution model (TVM + I + G) for the partial coxI mtDNA were run with four chains for 3 × 106, 2 × 106, and 2 × 106 generations, respectively. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualized using TreeView (Page 1996). In the ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 100 fast-step replicates.

2.5 Morphological matrix and mapping of morphological characters

Morphological characters used in morphospecies group delimitation were used to map them into the D2-D3 expansion segment of the 28S rRNA phylogenetic tree. Eleven characters were used for mapping onto the phylogenetic tree: L, the ratios a, b, c, and c′, vulva position, stylet length, lip region width, oral aperture to guiding ring distance, female tail length, and hyaline tail region length. Original species descriptions and newly obtained morphometric data were used as values. The values of the morphometric characters were coded as follows: L (1: < 1.5 mm; 2: ≥ 1.5–2.0 mm; and 3: ≥ 2 mm); a (1: ≤ 60; 2: 61–80 and 3: > 80); b (1: < 6.0; 2: ≥ 6.0–7.0; 3: > 7.0); c (1: < 60; 2: ≥ 60); c′ (1: < 1.1; 2: 1.1–1.5; 3: > 1.5–2; and 4: > 2); V (1: ≤50%; 2: 51–54%; 3: 55–58%; and 4: > 58%); stylet length (1: < 130 µm; 2: ≥ 130–147 µm; 3: > 147 µm); oral aperture to guiding ring distance (1: < 64 µm; 2: ≥ 64–78 µm; 3: > 78 µm); female tail length (1: < 27 µm; 2: ≥ 27–32 µm; 3: > 32 µm); hyaline tail region length (1: < 10; 2: ≥ 10). A new phylogenetic tree using a Bayesian approach was constructed using only one sequence for each species per clade, in case of different phylogenetic positions in the tree for a species, we kept the different sequences. Two approaches were used to map morphological characters (parsimony and Bayesian approaches). The criterion of parsimony was used to optimize character state evolution on the molecular consensus tree using MESQUITE 2.73 (Maddison and Maddison 2010). Ancestral characters with a clear pattern were selected for further studies. Ancestral character states were estimated according to their posterior probability distributions in a Bayesian approach using the program SIMMAP 1.5 (Bollback 2006). This program uses priors in morphological data analyses (Schultz and Churchill 1999). Morphology priors were calculated using a R script in the SIMMAP 1.5 program using R. expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI gene sequences.

3. Results

3.1 Systematics genus Xiphinema Cobb, 1913

Species determination of the *X. americanum*-group was based upon the integrative application of morphological, morphometric, and molecular methods in order to unravel potential cryptic species diversity. In addition, multivariate analyses were used on the studied populations, considered as variables, in order to verify the previously identified species.

3.1.1 *Xiphinema plesiopachtaicum* sp. nov.

Holotype:

Female extracted from soil samples collected from the rhizosphere of wild olive (*Olea europaea* ssp. *sylvestris*) in Coto Ríos, Jaén province, southern Spain (38°02′11.14″N, 2°54′11.27″E), by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine and deposited in the nematode collection at the Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR63-05).

Paratypes:

Female and juvenile paratypes extracted from soil samples collected from the rhizosphere of wild olive at Coto Ríos, Jaén province, southern Spain, were deposited in the following nematode collections: IASCSIC (collection numbers AR63-01–AR63-02, AR63- 04–AR63-12); two female paratypes at the Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT831), and two female paratypes at the USDA Nematode Collection, Beltsville, MD, USA (collection number T-6285p).

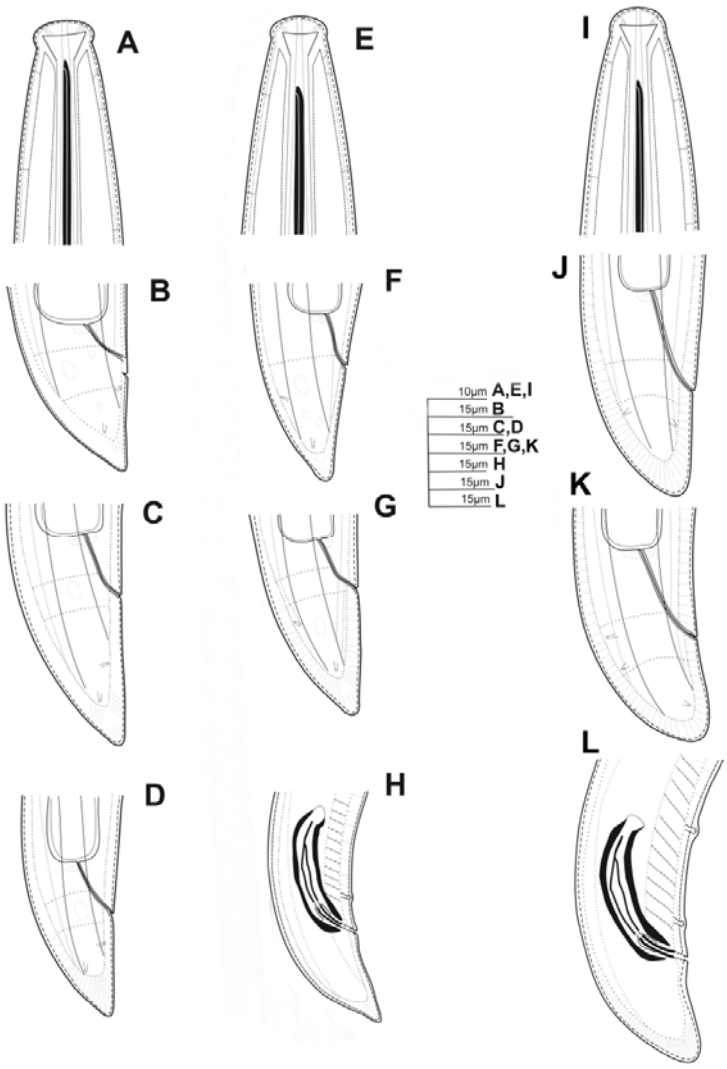

Figura 3.1*:* Line drawings of: A-D, ***Xiphinema plesiopachtaicum* sp. nov**.; E-H*,* ***Xiphinema vallense* sp. nov**.; I-L, ***Xiphinema astaregiense* sp. nov**. (I-L). A, E, I, female lip regions. B-D, F-G, and J-K, female tail regions. H, L, male tail regions.

Etymology:

The species epithet refers to a compound name from the Greek word *plesios* = near, and *pachtaicum*, the closest species of the genus *Xiphinema*.

Description of female:

Body medium-sized, forming a close C-shaped to open spiral when killed by heat. Body tapering very gradually towards the extremities. Cuticle smooth, finely striated transversely, 2.0 ± 0.2 (1.5–2.5) µm thick along body but thicker at tail tip (Table 3.2). Lip region flatly rounded, expanded, separated from rest of body by constriction, 9.5 ± 0.4 (8.5–10.5) µm wide. Amphidial fovea funnel-shaped, with slit-like aperture at constriction level, 6.6 ± 0.2 (6.5–7.0) µm wide. Pharynx consisting of an anterior slender narrow part, 238–350 µm long, extending to a terminal pharyngeal basal bulb well demarcated anteriorly, cylindrical, 73.4 ± 5.1 (63–84) µm long, 14.1 ± 1.1 (11.5–15.5) µm wide, occupying about one fifth to one third of the total pharyngeal length. Glandularium 65.0 ± 5.1 (56–78) µm long. Dorsal pharyngeal nucleus (DN) in anterior part of the bulb 12.4 ± 2.0 (10.4–16.0) % of basal bulb length, and subventrolateral nuclei (SVN) located around mid-bulb 53.3 ± 4.4 (49.5–61.7) % of basal bulb length (location of gland nuclei according to Loof and Coomans, 1972; Figure 3.1). Reproductive system amphidelphic, both branches equally developed; ovaries reflexed with symbiotic bacteria; uteri rather short (c. 138 µm long) without any differentiation. Vulva post-equatorial, transverse slit-like; vagina 11.3 ± 1.8 (9.0–15.0) µm long with short distal part and well developed proximally (Figure 3.2). Prerectum 449.0 ± 53.7 (411.0–487.0) µm long. Rectum 16.7 ± 3.4 (13.5–25.0) µm long. Tail short, but longer than anal body diameter (c′ = 1.3–1.7), dorsally convex-conoid, with pointed tip, and often with dorsal and occasionally dorsoventral depression at hyaline region level; two pairs of caudal pores present (Figure 3.2). Tail hyaline region 7.6 ± 1.3 (5.5–10.0) µm long.

Male:

Not detected.

Description of juveniles:

Only one juvenile stage was detected (fourth stage), which was morphologically similar to the female but from which juveniles differ by their size, longer and more tapering tails, and development of reproductive system.

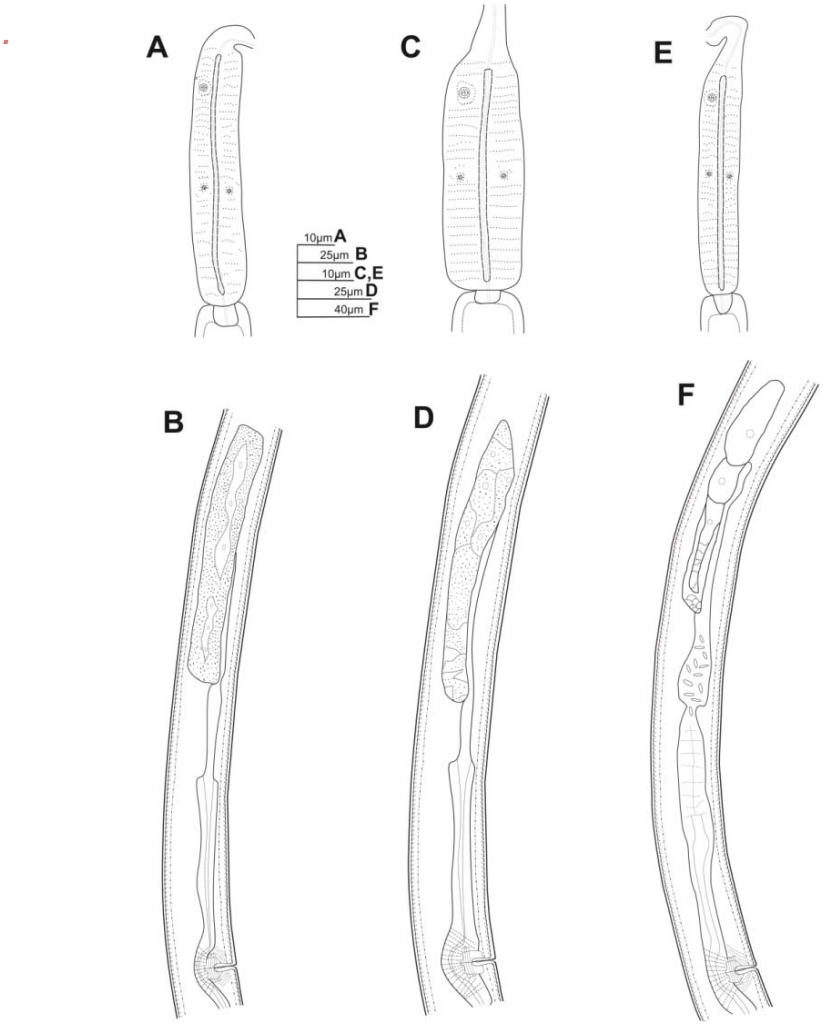

Figura 3.2: Line drawings of pharyngeal bulb and anterior genital branch of: A, B, ***Xiphinema plesiopachtaicum sp. nov.;*** C, D, **Xiphinema vallense *sp. nov.;*** E, F, **Xiphinema astaregiense *sp. nov***.

Diagnosis:

Xiphinema plesiopachtaicum sp. nov. is a parthenogenetic species characterized by a medium body size (1520–2078 µm); lip region flatly rounded, separated from the rest of the body by constriction; odontostyle and odontophore 83 and 48 µm long, respectively; V = 55–60%; female tail short (23.5–28.5 µm), dorsally convex-conoid, with pointed tip, and often with dorsal and occasionally dorsoventral depression at hyaline region level; c ratio of 62.5–88.7), c′ ratio of 1.3–1.7; and specific D2-D3 and ITS1-rRNA sequences deposited in GenBank with accession numbers KP268956–KP268958, and KP268973, respectively. Morphologically and morphometrically, *X. plesiopachtaicum* sp. nov. can be distinguished from the most similar species by a number of particular characteristics from its specific alphanumeric codes (exceptions are in parentheses): A 3(2), B 3, C 2(3), D 2, E 2, F2, G 2, H 1, I 1(2) sensu Lamberti *et al.* (2004) (Lamberti *et al.* 2004).

3.1.2 *Xiphinema vallense* sp. nov.

Holotype:

Female extracted from a loam soil from the rhizosphere of wild olive (*Olea europaea* ssp. *sylvestris*) in San José del Valle, Cádiz province, southern Spain (36°37′57.30″N, 5°46′20.00″W) by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine, and deposited in the nematode collection at IAS-CSIC (collection number AR55-06).

Paratypes:

Female, male, and juvenile paratypes extracted from the rhizosphere of wild olive (*Olea europaea* ssp. *sylvestris*) in San José del Valle, Cádiz province, southern Spain. Additional populations collected in Bolonia, Cádiz province, and Hinojos, Huelva province, both in southern Spain, associated with wild and cultivated olive, respectively, were deposited in the following nematode collections: IAS-CSIC (collection numbers AR55-01–AR55-05, AR55-07–AR55-011); two female paratypes deposited at the Royal Belgian Institute of

Natural Sciences, Brussels, Belgium (RIT830); and two female paratypes at the USDA Nematode Collection, Beltsville, MD, USA (collection number T-6287p).

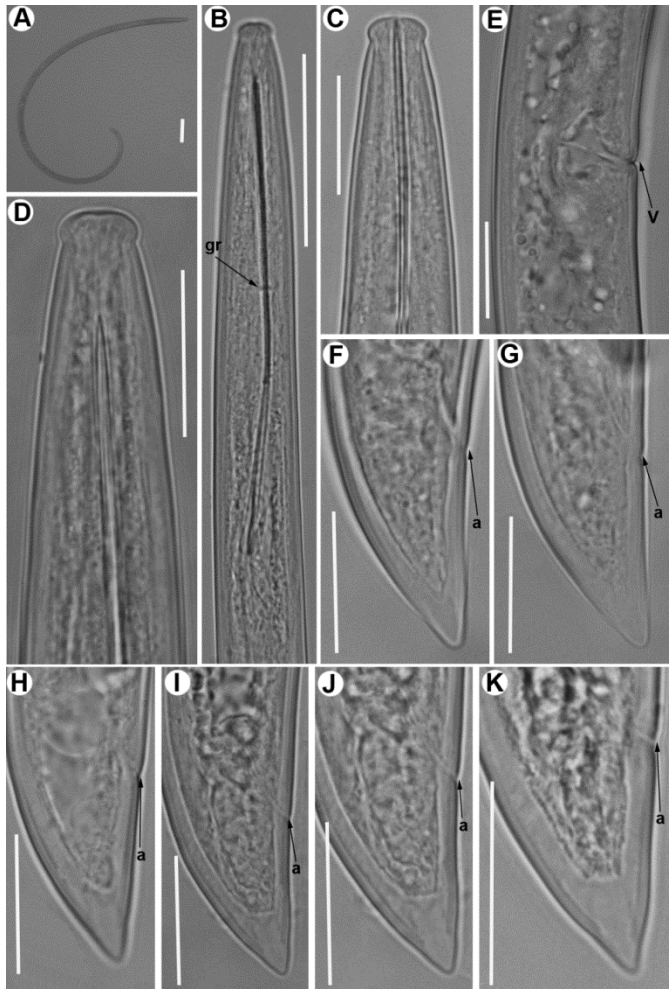

Figura 3.3: Light micrographs of ***Xiphinema plesiopachtaicum sp. nov.*** A, entire female. B, female neck region. C, D, female lip region. E, vulval region. F–K, female tail regions from different specimens showing the morphological variability. Abbreviations: a, anus; gr, guiding ring; V, vulva. Scale bars: A = 100 μ m; B–K = 20 μ m.

Table 3.2 Morphometrics of ***Xiphinema plesiopachtaicum* sp. nov.** from wild olive at Coto Ríos (Jaén, Spain)[a].

		Paratypes	
Characters/ratios [b]	**Holotype**	**Females**	**J4**
n	1	31	3
L	1917	1864 ± 124 (1520-2078)	1557 ± 23 (1533-1578)
a	62.9	64.0 ± 3.2 (57.3-70.2)	57.5 ± 4.1 (52.9-60.7)
b	6.0	6.8 ± 0.7 (5.6-8.2)	6.5 ± 1.4 (5.0-7.7)
c	75.2	73.1 ± 5.8 (62.5-88.7)	56.7 ± 2.6 (53.8-59.0)
c´	1.3	1.4 ± 0.1 (1.3-1.7)	1.5 ± 0.01 (1.4-1.5)
V or T	58.0	57.3 ± 1.0 (55.5-60.0)	-
G_1	16.3	12.9 ± 1.8 (9.7-16.3)	-
G_2	16.8	13.0 ± 1.8 (9.7-16.9)	-
Odontostyle length	84.0	82.6 ± 2.7 (77.0-89.0)	70.3 ± 2.5 (68.0-73.0)
Replacement odontostyle length	-	-	83.3 ± 2.6 (80.5-85.0)
Odontophore length	51.0	47.7 ± 3.0 (39.5-52.0)	44.2 ± 0.8 (43.5-45.0)
Lip region width	9.5	9.5 ± 0.4 (8.5-10.5)	8.8 ± 0.6 (8.5-9.5)
Oral aperture-guiding ring distance	69.0	69.4 ± 3.2 (63.0-76.5)	60.0 ± 2.8 (57.5-63.0)
Tail length	25.5	25.7 ± 1.3 (23.5-28.5)	27.5 ± 1.5 (26.0-29.0)
J	7.5	7.6 ± 1.3 (5.5-10.0)	7.7 ± 1.2 (6.0-10.0)

[a] Measurements are in µm and in the form: mean ± SD (range).
[b] a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Etymology:

The species epithet refers to the type locality, San José del Valle, where the species was detected.

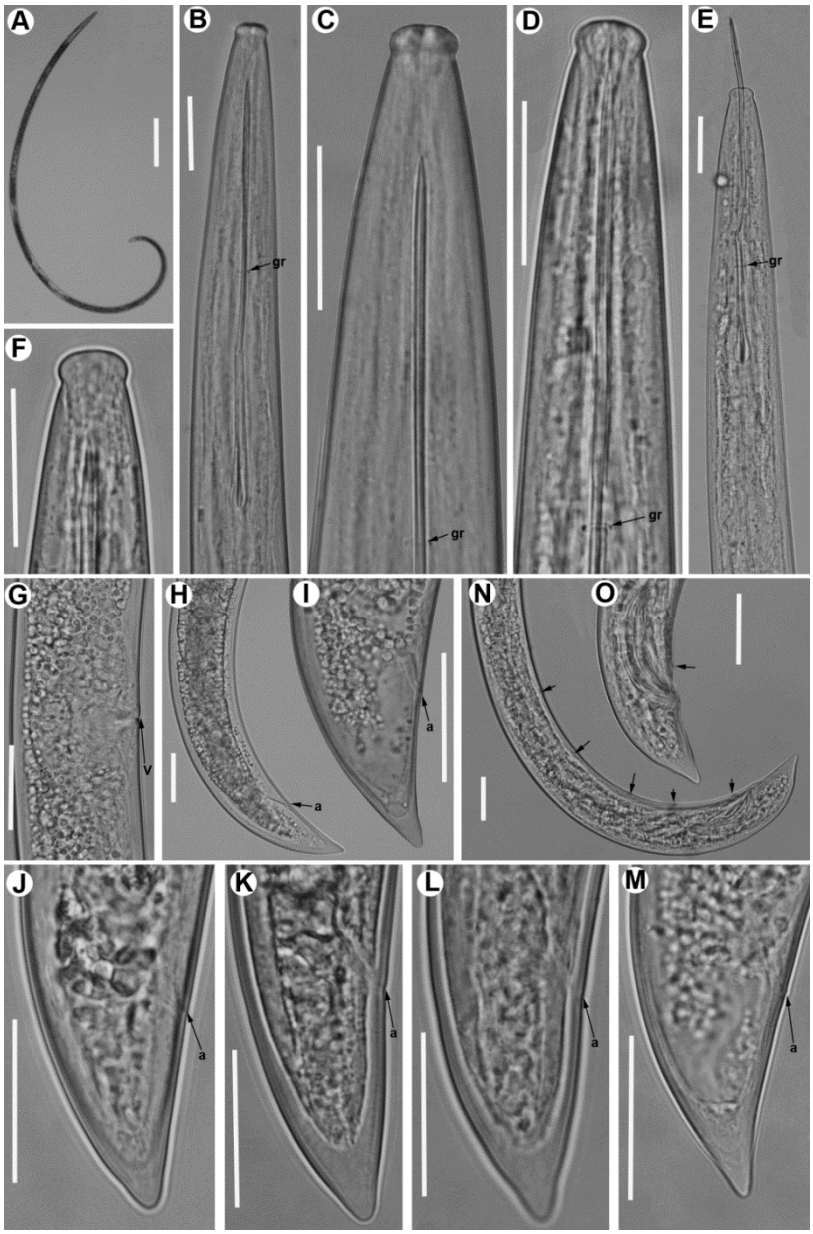

Figura 3.4: Light micrographs of ***Xiphinema vallense* sp. nov**. A, entire female. B, E, female neck region. C, D, F, female lip region. G, vulval region. H–M, female tail regions from different specimens showing the morphological variability. N–O, male tail, ventromedian supplements arrowed. Abbreviations: a, anus; gr, guiding ring; V, vulva. Scale bars: A = 200 µm; B–O = 20 µm.

Description of female:

Body medium to large sized, forming a close C-shaped to open spiral when killed by heat. Cuticle 2.0 ± 0.2 (1.5–2.5) µm thick along body but thicker at tail tip (Tables 3.3 and 3.4). Lip region widely rounded, separated from the rest of the body by constriction, 2.8 ± 0.3 (2.0–3.5) µm high. Amphidial fovea large, stirrup-shaped, with wide aperture occupying about 66–75% of corresponding lip region, as a straight transverse slit. Pharyngeal basal bulb 72.6 ± 7.8 (60–86) µm long and 14.5 ± 2.2 (11.0–18.0) µm wide, occupying about one-third to one-quarter of the total pharyngeal length (Figure 3.1). Glandularium 65.3 ± 6.8 (53–79) µm long. DN in anterior part of the bulb, 13.7 ± 1.8 (11.6–16.9) % of basal bulb length, and SVN located around mid-bulb, 51.3 ± 2.8 (47.4–55.0) % of basal bulb length (location of gland nuclei according to Loof and Coomans, 1972; Figures 3.1 and 3.2). Reproductive system amphidelphic, both branches equally developed; uteri rather short without any differentiation; ovaries without symbiontic bacteria (74–82 µm long). Vulva slit-like, clearly posterior to mid-body; vagina 11.4 ± 1.4 (9.0–14.0) µm long perpendicular to body axis; ovejector well developed, 15.5–21.0 µm wide, or 52.9–66.7% of corresponding maximum body diameter in lateral view (Figure 3.2). Prerectum 536.4 ± 36.9 (460–578) µm long. Rectum 18.7 ± 2.7 (14.5–23.5) µm long, or 0.6–1.2 times the anal body diameter. Tail short, dorsally convexconoid, with acute pointed tip, and often with dorsoventral depression at hyaline region level; two pairs of caudal pores present (Figure 3.2). Tail hyaline region 7.6 ± 0.6 (6.5–8.5) µm long (Figure 3.4).

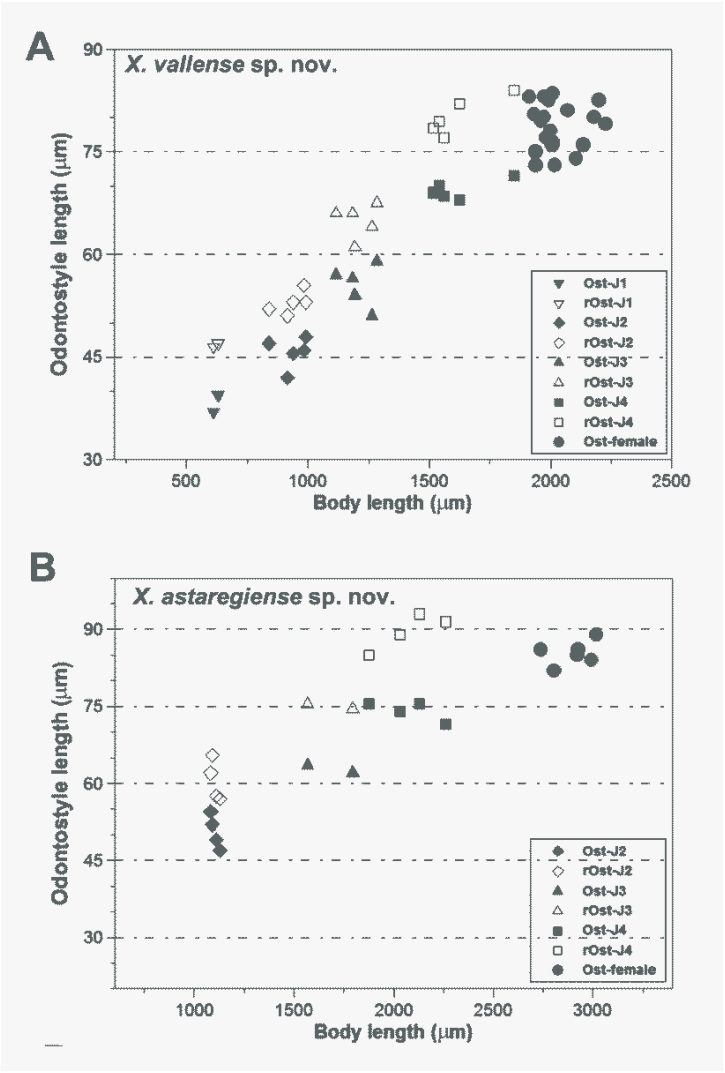

Figura 3.5: Relationship between body length and functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from first-stage juveniles (J1) to mature females of: A, ***Xiphinema vallense* sp. nov.** and B, ***Xiphinema astaregiense* sp. nov.**

Table 3.3 Morphometrics of ***Xiphinema vallense* sp. nov.** from wild olive at San José del Valle (Cádiz, Spain)[a].

		Paratypes					
Characters/ratios [b]	**Holotype**	**Females**	**Male**	**J1**	**J2**	**J3**	**J4**
n	1	21	1	2	5	6	7
L	1975	2019 ± 101 (1830-2228)	1861	620 ± 12.7 (611-629)	933 ± 62 (839-992)	1210 ± 60 (1117-1283)	1613 ± 111 (1517-1850)
a	73.1	68.9 ± 4.7 (61.6-79.1)	67,7	37.0 ± 1.5 (35.9-38.1)	43.7 ± 3.2 (38.1-46.1)	51.0 ± 3.4 (47.7-55.9)	60.5 ± 1.4 (57.8-61.7)
b	7.5	7.9 ± 0.9 (6.4-9.4)	6,6	4.2 ± 0.6 (3.7-4.6)	5.2 ± 0.8 (3.9-5.9)	5.1 ± 0.5 (4.4-5.8)	6.3 ± 0.8 (5.3-7.5)
c	70.5	73.4 ± 8.1 (58.2-86.3)	79,2	22.4 ± 1.9 (21.1-23.7)	34.2 ± 2.2 (31.7-37.4)	41.8 ± 2.2 (38.5-44.4)	55.4 ± 2.3 (53.2-60.2)
c´	1.6	1.6 ± 0.1 (1.4-1.7)	1,2	2.6 ± 0.01 (2.6-2.7)	2.1 ± 0.1 (2.0-2.1)	1.9 ± 0.1 (1.8-2.0)	1.8 ± 0.1 (1.6-1.9)
V or T	55.0	57.5 ± 1.2 (55.0-59.5)	-	-	-	-	-
G_1	10.1	8.7 ± 1.2 (7.2-9.1)	-	-	-	-	-
G_2	10.5	8.8 ± 1.0 (7.0-9.4)	-	-	-	-	-
Odontostyle length	83.0	79.0 ± 3.7 (73.0-85.5)	82.0	38.3 ± 1.8 (37.0-39.5)	45.7 ± 2.3 (42.0-48.0)	55.1 ± 2.9 (51.0-59.0)	68.1 ± 2.7 (63.0-71.5)
Replacement odontostyle length	-	-	-	46.8 ± 0.4 (46.5-47.0)	52.9 ± 1.7 (51.0-55.5)	66.4 ± 2.4 (61.0-67.5)	78.6 ± 3.8 (72.5-84.0)
Odontophore length	45.5	47.5 ± 2.5 (42.0-53.5)	43.0	26.5 ± 4.9 (23.0-30.0)	29.6 ± 3.0 (25.0-32.5)	38.5 ± 1.9 (36.5-41.0)	42.4 ± 2.4 (39.0-45.0)
Lip region width	8.0	8.5 ± 0.4 (8.0-9.0)	9.0	6.3 ± 0.4 (6.0-6.5)	2.5 ± 0.4 (2.0-3.0)	7.4 ± 0.5 (7.0-8.0)	7.9 ± 0.4 (7.5-8.5)
Oral aperture-guiding ring distance	73.0	69.5 ± 3.7 (62.0-75.5)	64.0	32.5 ± 2.1 (31.0-34.0)	39.1 ± 2.4 (35.0-41.0)	48.3 ± 3.5 (44.0-53.0)	60.1 ± 2.0 (56.0-62.5)
Tail length	28.0	27.8 ± 2.8 (22.5-34.0)	23.5	27.8± 1.8 (26.5-29.0)	27.3 ± 1.5 (26.5-30.0)	29.0 ± 1.5 (27.5-31.0)	29.1 ± 2.2 (26.5-33.5)
J	7.5	7.6 ± 0.6 (6.5-8.5)	8.0	5.0 ± 0.0 (5.0-5.0)	5.4 ± 0.9 (4.5-6.5)	6.5 ± 0.5 (6.0-7.0)	6.3 ± 0.3 (6.0-6.5)
Spicules	-	-	38.0	-	-	-	-
Lateral accessory piece	-	-	8.0	-	-	-	-

[a] Measurements are in µm and in the form: mean ± SD (range).

[b] a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Description of male:

Extremely rare, only one male was found, which was similar to the female except for a rather coiled posterior region. Testes well developed, 41.6% of body length. Spicules well sclerotized, ventrally curved. Lateral guiding pieces about one-fifth of the length of the spicules. A precloacal pair of supplements 8.0 µm anterior to cloacal opening and a row of six single ventromedian supplements, located anterior to the retracted spicule (Figure 3.4).

Description of juveniles:

All four juvenile stages (first, second, third, and fourth stage) were found, and were similar to adults, except for their smaller size, longer tails, and absence of sexual characteristics. Tail becoming progressively shorter and stouter in each moult; different development stages distinguishable by relative lengths of body and functional and replacement odontostyle (Figure 3.5; Tables 3.3 and 3.4).

Diagnosis:

Xiphinema vallense sp. nov. is a amphimictic species characterized by a medium to large body size (1830–2228 µm); lip region widely rounded, separated from the rest of the body by a constriction; odontostyle and odontophore 79 and 48 µm long, respectively; V = 55–59%; female tail 22.5–34.0 µm long, dorsally convex-conoid, often with dorsoventral depression at hyaline region level, with accurate pointed tip; c ratio of 58.2–86.3; c′ ratio of 1.4–1.7; and specific specific D2-D3, and ITS1-rRNA sequences deposited in GenBank under accession numbers KP268959–KP268961 and KP268974, respectively.

Table 3.4 Morphometrics of ***Xiphinema vallense* sp. nov.** from wild and cultivated olive at Bolonia (Cádiz province) and Hinojos (Huelva province), Spain, respectively[a].

	Bolonia	Hinojos
Characters/ratios [b]	**Females**	**Females**
n	4	4
L	1857 ± 60 (1795-1912)	1903 ± 136 (1713-2011)
a	57.6 ± 2.0 (55.1-59.5)	63.5 ± 5.2 (56.2-67.4)
b	7.2 ± 0.4 (6.9-7.8)	8.8 ± 1.0 (7.3-9.7)
c	67.6 ± 9.0 (67.2-81.1)	75.0 ± 4.7 (68.5-78.9)
c´	1.5 ± 0.1 (1.5-1.6)	1.4 ± 0.03 (1.4-1.5)
V or T	59.1 ± 1.5 (57.0-60.5)	58.1 ± 1.3 (57.0-59.5)
G_1	8.7 ± 0.4 (8.4-9.0)	11.6 ± 2.0 (10.2-13.0)
G_2	9.5 ± 0.8 (8.9-10.0)	11.9 ± 2.5 (10.2-13.7)
Odontostyle length	78.3 ± 2.9 (75.5-81.0)	75.1 ± 1.4 (73.0-76.0)
Odontophore length	48.3 ± 2.0 (46.5-51.0)	49.6 ± 2.8 (46.0-52.5)
Lip region width	8.1 ± 0.3 (8.0-8.5)	9.1 ± 0.3 (9.0-9.5)
Oral aperture-guiding ring distance	67.3 ± 3.2 (64.0-71.5)	63.1 ± 2.7 (59.5-66.0)
Tail length	27.8 ± 3.0 (23.5-30.5)	25.4 ± 0.3 (25.0-25.5)
J	7.6 ± 0.3 (7.5-8.0)	7.5 ± 0.9 (6.5-8.5)

[a] Measurements are in µm and in the form: mean ± SD (range).
[b] a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Morphologically and morphometrically, *X. vallense* sp. nov. can be distinguished from the most similar species by a number of particular characteristics from its specific alphanumeric codes (exceptions are in parentheses): A 2(3), B 3(4), C 3(2), D 2(3), E 2(3), F2(1), G 2, H 1, I 2(1) sensu Lamberti et al. (2004).

3.1.3 *Xiphinema astaregiense* sp. nov.

Holotype:

Female extracted from soil samples collected from rhizosphere of unidentified grasses (Graminaceae) in Jerez de la Frontera, Cadiz province, southern Spain, (36°46′31.36″N, 6°15′15.67″W) by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine, and deposited in the nematode collection at IAS-CSIC (collection number J174-010).

Paratypes:

Female, male, and juvenile paratypes extracted from the rhizosphere of unidentified grasses (Graminaceae) in Jerez de la Frontera, Cadiz province, southern Spain, were deposited in the following nematode collections: IAS-CSIC (collection numbers J174-02, J174-03, J174-04); one female and one male at the Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT832); and one female at USDA Nematode Collection (T-6288p).

Etymology:

The species epithet refers to the old Latin name of the type locality, *Asta Regia* (Jerez de la Frontera), where the nematode was detected.

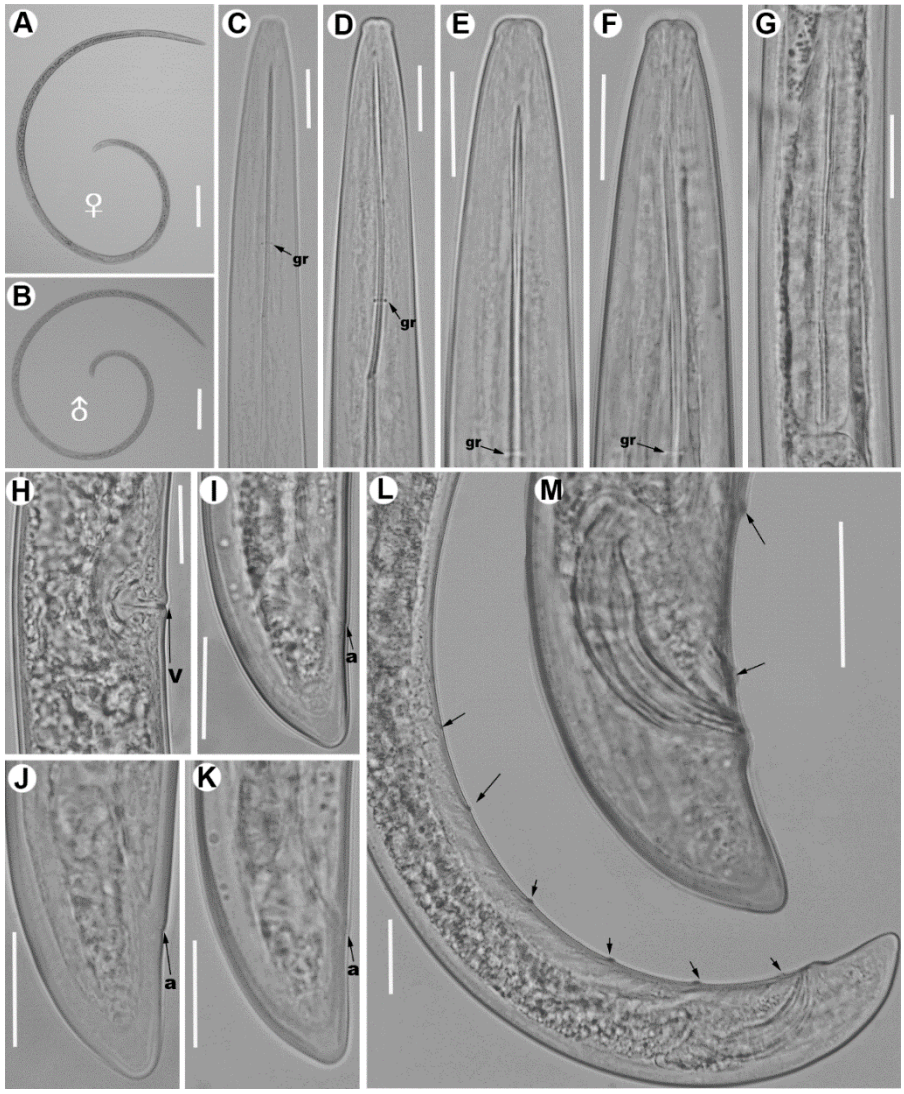

Figura 3.6: Light micrographs of ***Xiphinema astaregiense* sp. nov.** A, B, entire female and male, respectively. C–F, female neck region. G, pharyngeal bulb. H, vulval region. I–K, female tail regions from different specimens showing the morphological variability. L, M, male tail region, ventromedian supplements arrowed. Abbreviations: a, anus; gr, guiding ring; *V*, vulva. Scale bars: A, B = 200 µm; C–M = 20 µm.

Table 3.5 Morphometrics of ***Xiphinema astaregiense* sp. nov.** from grasses at Jerez de la Frontera (Cádiz, Spain)[a].

Characters/ratios [b]	Holotype	Paratypes				
		Females	Males	J2	J3	J4
n	1	6	7	4	2	4
L	2740	2981 ± 108 (2740-3018)	2649 ± 95 (2577-2840)	1102 ± 15 (1083-1111)	1681 ± 161 (1567-1795)	2075 ± 161 (1878-2261)
a	76.1	72.4 ± 3.7 (66.8-76.1)	75.5 ± 4.1 (69.6-83.5)	49.5 ± 6.5 (43.3-55.6)	68.0 ± 7.5 (62.7-73.3)	69.8 ± 4.4 (64.5-75.1)
b	7.8	9.1 ± 1.0 (7.8-10.1)	9.0 ± 1.7 (7.4-11.8)	6.4 ± 0.4 (5.8-6.7)	7.2 ± 1.3 (6.2-8.1)	7.6 ± 0.8 (6.5-8.5)
c	114.2	123.2 ± 7.8 (112.2-129.9)	107.5 ± 6.3 (100.2-117.4)	38.8 ± 3.4 (35.1-41.1)	66.0 ± 8.2 (60.3-71.8)	80.1 ± 8.2 (74.7-92.3)
c´	1.0	1.0 ± 0.1 (0.9-1.1)	1,0 ± 0.1 (1.0-1.2)	2.0 ± 0.1 (1.9-2.2)	1.5 ± 0.01 (1.5-1.6)	1.4 ± 0.1 (1.3-1.4)
V or T	55.5	56.9 ± 1.4 (55.5-59.0)	-	-	-	-
G_1	11.3	11.5 ± 1.5 (10.6-12.2)	-	-	-	-
G_2	10.8	11.4 ± 0.4 (10.8-11.9)	-	-	-	-
Odontostyle length	86.0	85.3 ± 2.3 (82.0-89.0)	87.6 ± 2.7 (84.0-91.0)	50.6 ± 2.8 (49.0-54.5)	62.8 ± 1.1 (62.0-63.5)	74.1 ± 1.9 (71.5-75.5)
Replacement odontostyle length	-	-	-	60.5 ± 4.0 (57.5-65.5)	75.0 ± 0.7 (74.5-75.5)	89.6 ± 3.5 (85.0-93.0)
Odontophore length	55.5	54.1 ± 1.4 (52.0-55.5)	51.9 ± 1.9 (49.0-54.5)	30.5 ± 2.1 (29.0-33.0)	38.5 ± 7.8 (33.0-44.0)	45.0 ± 2.2 (43.0-48.0)
Lip region width	9.0	9.3 ± 0.7 (8.5-10.5)	9.1 ± 0.2 (9.0-9.5)	7.3 ± 0.3 (7.0-7.5)	7.8 ± 0.4 (7.5-8.0)	8.0 ± 0.4 (7.5-8.5)
Oral aperture-guiding ring distance	76.5	73.2 ± 3.7 (70.0-79.0)	75.1 ± 1.3 (73.0-77.0)	42.3 ± 2.1 (40.0-44.0)	53.3 ± 1.1 (52.5-54.0)	64.0 ± 2.2 (62.0-67.0)
Tail length	24.0	23.6 ± 0.9 (22.5-25.0)	24.7 ± 1.5 (22.0-26.0)	28.6 ± 2.2 (27.0-31.0)	24.8 ± 0.4 (24.5-25.0)	26.0 ± 1.9 (24.5-28.5)
J	8.0	8.2 ± 0.5 (7.5-9.0)	7.4 ± 0.5 (7.0-8.0)	7.6 ± 0.3 (7.5-8.0)	7.3 ± 1.8 (6.0-8.5)	7.8 ± 0.6 (7.0-8.5)
Spicules	-	-	47.1 ± 2.0 (43.0-49.0)	-	-	-
Lateral accessory piece	-	-	10.1 ± 0.5 (9.5-11.0)	-	-	-

[a] Measurements are in µm and in the form: mean ± SD (range).
[b] a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Description of female:

Body large-sized, habitus coiled in a more or less closed C-shaped to open spiral when killed by heat. Body tapering very gradually toward the posterior extremity and more abruptly in the anterior region. Cuticle finely striated transversally, 2.0–2.5 µm thick along body but thicker at tail tip (Table 3.5, Figure 3.6). Lip region anteriorly flattened, laterally rounded, separated from the rest of body by a depression, 8.5–10.5 µm wide and 4.0–5.5 µm high. Amphidial fovea large, stirrup-shaped with slit-like aperture, occupying c. 77.0% of corresponding lip region width. Pharynx consisting of an anterior slender narrow part, 285–364 µm long, extending to a terminal pharyngeal basal bulb well demarcated anteriorly, cylindrical, 93.7 ± 5.3 (85–101) µm long, 17.2 ± 2.5 (15.5–20.0) µm wide, occupying about one-quarter to one-third of the total pharyngeal length (Figure 3.1). Glandularium 76.5–85.0 µm long. DN in anterior part of the bulb, 18.5 ± 3.2 (16.2–20.7) % of basal bulb length, and SVN located around mid-bulb, 48.8 ± 0.4 (48.5–49.1) % of basal bulb length (location of gland nuclei according to Loof and Coomans, 1972). Reproductive system amphidelphic, both branches equally developed; ovaries reflexed without symbiontic bacteria; uteri often with spindle-shaped sperm cells 2.0–3.5 µm long, without any differentiation. Vulva slit like, clearly posterior to mid-body; vagina 14.0 ± 1.5 (12.5–16.0) µm long perpendicular to body axis; ovejector well developed, 22.0–28.5 µm wide, or 58.3–69.1% of maximum body diameter in lateral view (Figure 3.6). Prerectum often indistinct. Rectum 19.6 ± 1.8 (18.0–22.5) µm long, or 0.7–1.9 times the anal body diameter. Tail short, dorsally convex-conoid, with curvature essentially dorsal with conoid-rounded terminus, bearing two and three caudal pores (Figure 3.2). Tail hyaline region about one-third of the tail length.

Description of male:

Common (almost as frequent as female, c. 45%). Morphologically similar to female except for genital system, but with posterior part of the body more curved with greater curvature in posterior part of body (Figure 3.3). Testis well developed, containing numerous spindle-shaped sperms. Spicules well sclerotized, ventrally curved with bifid lateral guiding pieces 10.1 ± 0.5 (9.5–11.0) µm long (Figure 3.6). A preanal pair of supplements 9.0 to 11.0 µm

anterior to cloacal opening and a row of six to seven single ventromedian supplements, located anterior to the spicule region (Figure 3.6).

Description of juveniles:

All juvenile stages, except for the first, were detected. They are generally similar to adults, except for their smaller size, longer tails, and absence of sexual characteristics. Tail becoming progressively shorter and stouter in each moult; different developmental stages distinguishable by relative lengths of body and functional and replacement odontostyle (Figure 3.5, Table 3.5).

Diagnosis:

Xiphinema astaregiense sp. nov. is a bisexual species characterized by a large body size (2740–3018 µm); lip region anteriorly flattened and laterally rounded, separated from the body by a depression; odontostyle and odontophore 85 and 54 µm long, respectively, the latter with well-developed flanges; V = 55–59%; length of female tail 22.5–25.0 µm, relatively short, convex-conoid with curvature essentially dorsal and conoid-rounded terminus; c ratio (112.2–129.9), c′ ratio (0.9–1.1); and specific D2-D3, ITS1-rRNA, and coxI sequences deposited in GenBank with accession numbers KP268955, KP268972, and KP268977, respectively.

Morphologically and morphometrically, *X. astaregiense* sp. nov. can be distinguished from the most similar species by a number of particular characteristics from its specific alphanumeric codes (exceptions are in parentheses): A 3, B 3, C 1(2), D 3, E 3, F2, G 2, H 2, I 1 sensu Lamberti et al. (2004).

3.1.4 Morphology and morphometrics of species of *the Xiphinema americanum*-group

The morphological and morphometric data as well as molecular delineation for *X. duriense* Lamberti *et al*. 1993, *X. incertum* Lamberti et al. 1983, *X. opisthohysterum* Siddiqi 1961, *X. pachtaicum* (Tulaganov, 1938) Kirjanova 1951, X. parapachydermum Gutiérrez-Gutiérrez *et al.* 2012, and *X. rivesi*

Dalmasso 1969, were previously studied and compared with original descriptions and paratype specimens within previous studies on the identification and molecular phylogeny of the *X. americanum*-group in southern Spain (Figure S3.13, Talbe S3.10) (Gutiérrez-Gutiérrez *et al.* 2011b, 2012). The new records of these species from olive in Seville and Huelva provinces and also in Almeria province presented here extend the geographical distribution of these species in southern Spain (Gutiérrez-Gutiérrez *et al.* 2012). For these species only the D2-D3 sequences have been reported here for these samples. For other known species studied, representing the first molecular characterization and/or new records for olive or for Spain, a brief description and a morphometric comparison with previous records is provided below.

3.1.4.1 *Xiphinema brevisicum* Lamberti *et al*. 1994

The Spanish population of this species is characterized by a coiled body habitus forming an open C when killed by heat, lip region expanded and offset from the body by a constriction, female reproductive system amphidelphic with two equally developed genital branches, tail elongated-conoid, slightly curved ventrally, two caudal pores on each side. Male frequent, habitus more coiled than female. Tail elongated with pointed tip, ventrally curved with four to five ventromedian supplements preceding the adanal pair. The morphology and morphometrics of the Spanish population agree closely with those of the original description from grapevine and natural vegetation in Portugal by Lamberti *et al.* (1994) (Figure S3.14, Table S3.10), except for lower a and c ratios in females (average 79.5, 47.8 *vs* average 88.5, 56.8, respectively). Nevertheless, these differences further expand the intraspecific variation but do not exceed that reported by *Lamberti et al.* (1994). This species was reported from north-western Spain by Abelleira, Picoaga and Mansilla (2008) but no morphometric or molecular characterization was provided. These data indicate that this species may be an Iberian endemic species associated with cultivated and wild plants as suggested by Peña-Santiago et al. (2006). The alphanumeric codes for *X. brevisicum* to be applied to the polytomic identification key for

the *X. americanum*-group species by Lamberti *et al*. (2004) are (exceptions are in parentheses): A 1, B 2, C 4, D 1, E 3(2), F 2, G 2, H 1, I 3.

3.1.4.2 *Xiphinema luci* Lamberti and Bleve-Zacheo 1979

The Spanish population of this species is characterized by a body ventrally curved in an open C when killed by heat, lip region flat-rounded, and separated from the body by a depression (Figure S3.15, Table S3.10). Female reproductive system amphidelphic with two equally developed genital branches and absence of uterine differentiation, ovary contains symbiontic bacteria, and vulva a transverse slit located slightly posterior to mid-body. Tail short, broadly convex-conoid with bluntly rounded terminus and bearing three pairs of caudal pores (Figure S3.15). Males not found. The morphology and morphometrics of this population closely agree with the original description from celery in Diourbel, Senegal (Lamberti and Bleve-Zacheo 1979), and a population from common screw pine (*Pandanus utilis* Bory.) in the Botanical Garden of Dakar, Senegal (Faye *et al.* 2012), except for a lower c ratio [60.9–68.6 vs. 54.0–81.0 (Lamberti and Bleve-Zacheo 1979), 63.0–87.0 (Faye *et al.* 2012)]. This difference should be regarded as geographical intraspecific variation. The species has been also reported in Florida but no morphometrics were provided (Robbins 1993). The alphanumeric codes for *X. luci* to be applied to the polytomic identification key for the *X. americanum*-group species by Lamberti *et al*. (2004) are (exceptions are in parentheses): A 3(4), B 2, C 2, D 2, E 2(3), F 1, G 1, H 2, I 2. morphometric comparison with previous records is provided below.

3.1.4.3 *Xiphinema madeirense* Brown *et al*. 1992

The Spanish population of this species is characterized by a relatively long body (*ca* 2 mm), forming an open coiled spiral when killed by heat, lip region expanded and clearly offset from the body by a depression, and a long odontostyle (92.5–100.5 µm long). Female reproductive system with two equally developed genital branches and uterine differentiation absent, vulva slit-like, posterior to mid-body, and vagina occupying about half of the

body width. Tail conoid-elongate, curved ventrally, with almost pointed terminus, and bearing two pairs of caudal pores. Male not found. The morphology and morphometrics of this population agree with the original description (Brown *et al.* 1992) and other populations from Portugal (Lamberti *et al.* 1993, 1994) (Figure S3.16, Table S3.10).

This work represents the first report of this nematode species in Spain, although it has been described previously from the rhizosphere of bay laurel (*Laurus nobilis* L.) in Queimadas, Santana, on the island of Madeira, where it seems to occur in natural habitats (Brown *et al.* 1992). It is also quite common and widespread in grapevines, fallow soil, and the rhizosphere of peach or hop in northern and central Portugal (Lamberti *et al.* 1994). The alphanumeric codes for *X. madeirense* to be applied to the polytomic identification key for the *X. americanum*-group species by Lamberti *et al*. (2004) are (exceptions are in parentheses): A4, B 3, C 3(4), D 2, E 3, F 2, G 2, H 1, I 3.

3.2 Molecular characterization *of* *Xiphinema plesiopachtaicum* sp. nov., *Xiphinema vallense* sp. nov., *Xiphinema astaregiense* sp. nov., and other *Xiphinema americanum*-subgroup species

Amplification of the D2-D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI regions from the three new and the previously known *X. americanum*-group species yielded single fragments of approximately 800, 1100, and 400 bp, respectively, based on gel electrophoresis. D2-D3 sequences of *X. plesiopachtaicum* sp. nov., *X. vallense* sp. nov., and *X. astaregiense* sp. nov. matched well with many species of the *X. americanum*-group spp. deposited in GenBank, although for other species were clearly dissimilar (Table 3.6). These sequences were closed to *X. duriense*, *X. incertum*, *X. luci*, *X. madeirense*, *X. pachtaicum*, and *X. parapachydermum* (Table 3.6). Intraspecific variation in D2-D3 segments was detected amongst three specimens of *X. plesiopachtaicum* sp. nov., consisting of one to three nucleotides (99% similarity) and one indel (0.14%). Similarly, intraspecific variation in D2-D3 segments was detected amongst the three studied populations (two from wild olive, and one from cultivated olive) of *X. vallense* sp. nov., consisting of zero to five

nucleotides (99% similarity) and no indels. ITS1 rRNA sequences from *X. plesiopachtaicum* sp. nov. (KP268973), *X. vallense* sp. nov. (KP268974), *X. astaregiense* sp. nov. (KP268972), and *X. madeirense* (KP268976) showed low homology with the majority of ITS1 sequences deposited in GenBank, except for X. parapachydermum (JQ990045) and *X. pachtaicum* (HM921337, AY430178) (Table 3.6). ITS1 from *X. luci* (KP268975) matched closely with the *X. americanum*-group spp. deposited in GenBank. This sequence was 98, 98, and 95% similar to *Xiphinema oxycaudatum* (AY359859), *Xiphinema peruvianum* (GQ231531), and *X. americanum* (KF748291), respectively; and varied by ten, ten, and 25 nucleotides, respectively. Finally, repeated difficulties were experienced with the partial coxI sequences from *X. plesiopachtaicum* sp. nov. and *X. vallense* sp. nov. and they could not be sequenced. The partial coxI sequence from *X. astaregiense* sp. nov. (KP268977) was clearly different to the coxI sequences of the *X. americanum*-group deposited in GenBank, being 79 to 75% similar to some of them, such as X. pachtaicum (HM921378), *X. incertum* (JQ990058), and *Xiphinema incognitum* (AM086705); and varied by 83, 98, and 86 nucleotides, respectively.

Table 3.6 Identity matrix, proportion (%) of identical residues between (indels included) rDNA sequences among *Xiphinema* species. Above diagonal D2-D3 expansion segments of 28S rRNA and below diagonal ITS1 region*.

	Xiphinema spp.								
Xiphinema spp.	**1**	**2**	**3**	4	5	6	7	8	9
1. *X. astaregiense* sp. nov.*		96	85	81	50	90	85	85	85
2. *X. plesiopachtaicum* sp. nov.	50		87	82	49	91	86	85	85
3. *X. vallense* sp. nov.	30	34		82	49	80	74	76	83
4. *X. madeirense*	35	39	34		51	77	72	72	80
5. *X. luci*	30	37	30	47		45	54	54	53
6. *X. incertum*	44	39	-	-	31		80	83	80
7. *X. parapachydermum*	59	65	37	42	33	38		94	78
8. *X. pachtaicum*	63	43	-	33	-	59	50		78
9. *X. pachydermum*	-	-	-	-	-	-	-	-	

* Accession numbers (D2-D3, ITS1, respectively) for each *Xiphinema* spp.: **1= KP268955, KP268972**; **2= KP268956, KP268973**; **3= KP268959, KP268974**; 4= **KP268966, KP268976**; 5= **KP268965, KP268975**; 6= JQ990031, JQ990044; 7= JQ990035, JQ990045; 8= JQ990033, HM924337; 9= AY601608, -.

(-) Sequences not available or comparison not carried out because of low homology between sequences.Newly obtained sequences are in bold letters.

3.3 Multivariate analyses of *Xiphinema plesiopachtaicum* sp. nov., *Xiphinema vallense* sp. nov., and other *Xiphinema pachtaicum*-species complex

In the factor analysis, the first four factors (SS loadings > 1) accounted for 73.10% of the total variance in the morphometric characters of *X. plesiopachtaicum* sp. nov. and *X. vallense* sp. nov. as well as *X. incertum*, *X. madeirense*, *X. pachtaicum*, and *X. parapachydermum* (Table 3.7). Table 3.7 includes the SS loadings for the four factors extracted, which were a linear combination of all characters in the analysis. The eigenvectors for each character were used to interpret the biological meaning of the factors. Factor 1 was dominated by high positive weights (eigenvector > 0.88) for stylet length, and oral aperture-guiding ring distance (Table 3.7, Figure 3.7A-C). Factor 2 was dominated by a high positive weight (eigenvector = 0.73) for c′ ratio, and high negative weights (eigenvector < −0.67) for lip region width and c ratio (Table 3.7, Figure 3.7A, D), thereby relating this factor to lip region and female tail shape. Factor 3 was dominated only by positive weights (eigenvector > 0.85) for body length and a ratio (Table 3.7, Figure 3.7B, D), relating this factor to the overall size and shape of nematode populations. Finally, Factor 4 was dominated by positive and negative weights for b ratio (eigenvector = 0.70) and V = (eigenvector = −0.79), respectively (Table 3.7, Figure 3.7C). Tail and hyaline region length were not highly associated with any of the factors extracted but, to a lesser extent, showed positive weights for Factors 1 and 2 (eigenvector > 0.63) for tail length, and for Factor 2 (eigenvector = 0.55) in the case of hyaline region length (Table 3.7, Figure 3.7A-D). The results of the factor analysis were represented graphically in Cartesian plots in which *Xiphinema* populations were projected on the plane of the x- and y-axes, respectively, as pairwise combinations of Factors 1 to 4 (Figure 3.8A-D). With few exceptions, populations of each species were projected close to each other, except for *X. incertum*, which showed a wide distribution for all combinations of factors owing to their wide morphometric variation amongst populations (Table S3.11).

Table 3.7 Eigenvector and SS loadings of factor derived from nematode morphometric characters for ***Xiphinema plesiopachtaicum* sp. nov.**, ***Xiphinema vallense* sp. nov.**, one population of *X. madeirense*, two populations of *X. incertum*, three populations of *X. parapachydermum*, and seven populations of *X. pachtaicum*[a].

	Principal component			
Character[b]	**F1**	**F2**	**F3**	**F4**
Body length (L)	0.274	-0.158	0.855	-0.148
A	-0.095	0.097	0.884	0.103
B	-0.128	0.011	0.100	-0.795
C	-0.341	-0.670	0.561	0.038
c´	0.303	0.727	0.282	0.206
V	-0.329	0.100	0.138	0.701
Stylet length	0.883	0.065	-0.037	-0.068
Oral aperture-guiding ring distance	0.891	0.096	0.077	0.022
Lip region width	0.277	-0.711	0.036	0.156
Tail length	0.644	0.635	0.080	-0.145
Hyaline region length	0.155	0.555	-0.181	0.233
SS loadings	2.506	2.251	1.983	1.304
% of total variance	22.80	20.50	18.80	11.90
Cumulative % of total variance	22.80	43.20	61.30	73.10

[a] Based on 31 female specimens of ***Xiphinema plesiopachtaicum* sp. nov.**, 21 female specimens of ***Xiphinema vallense* sp. nov.**, 9 female specimens of one population of *X. madeirense*, 6 and 7 female specimens of two populations of *X. incertum*, 10, 8, and 7 female specimens of three populations of *X. parapachydermum*, and 10, 9, 9, 10, 10, 6 and 10 female specimens of seven populations of *X. pachtaicum*. All populations from Spain. Values of morphometric factors 1 to 4 (eigenvector >0.67) are underlined.

[b] Morphological and diagnostic characters according to Lamberti and Ciancio (1993) with some inclusions.

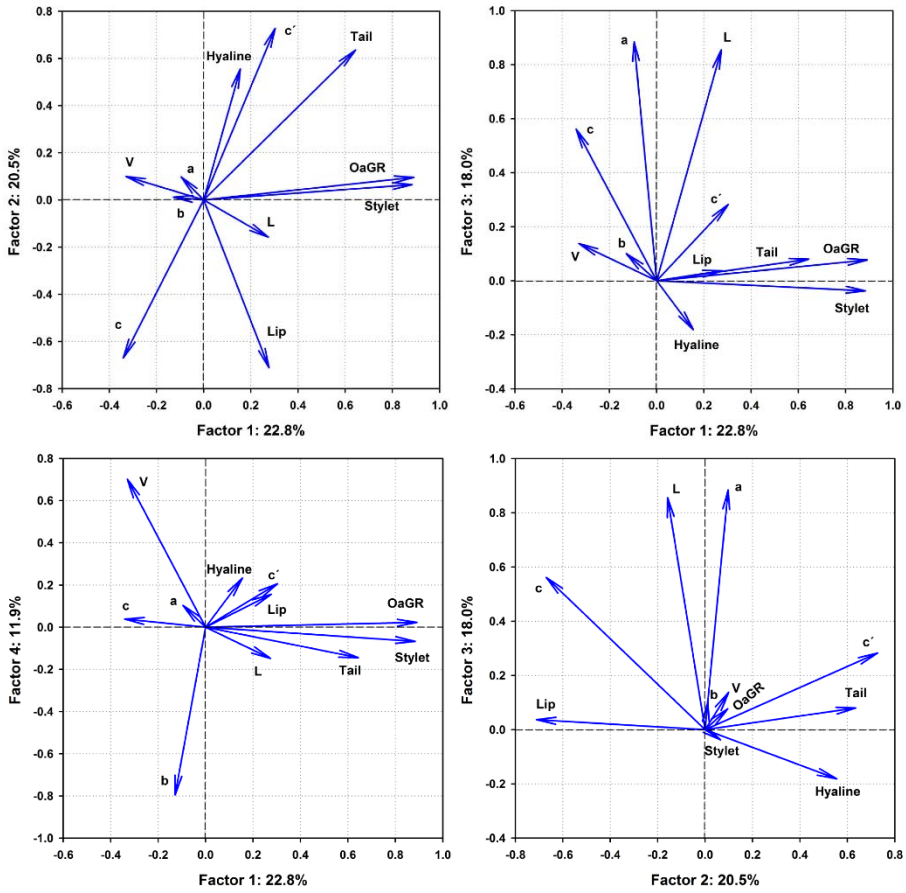

Figura 3.7: Factor analysis of 11 morphometric characters used to characterize ***Xiphinema plesiopachtaicum* sp. nov.**, ***Xiphinema vallense* sp. nov.**, and *Xiphinema pachtaicum*-subgroup species. Left-hand side of panels: projection of morphometric characters on the plane of factors 1 and 2 (A), 1 and 3 (B), 1 and 4 (C), and 2 and 3 (D). Abbreviations: L, body length; V, (distance from anterior end to vulva/body length) × 100; OaGR, oral aperture-guiding ring distance; Lip, lip region width; Tail, female tail length; Hyaline, hyaline region length; a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c′, tail length/body width at anus.

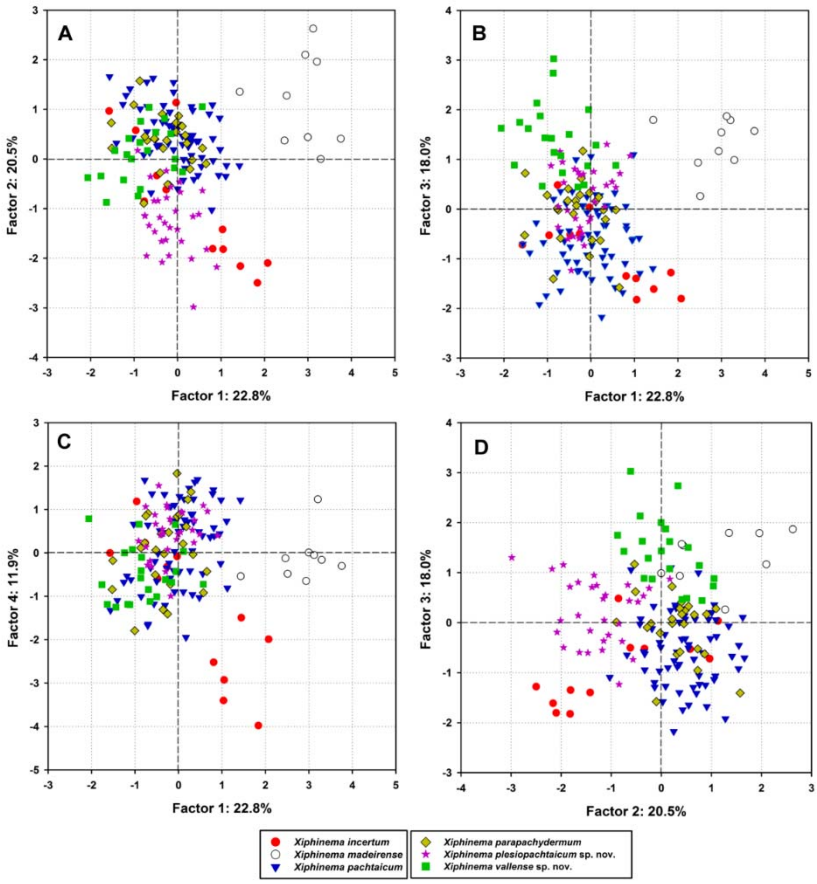

Figura 3.8: Factor analysis of 11 morphometric characters used to characterize ***Xiphinema plesiopachtaicum* sp. nov**., ***Xiphinema vallense* sp. nov**., and *Xiphinema pachtaicum*-subgroup species. Projection of Xiphinema americanum-group species on the plane of factor 1 and 2 (A), 1 and 3 (B), 1 and 4 (C), and 2 and 3 (D).

According to their relative position along the x-axis (Factor 1) in Figure 3.7A-C, the stylet length and oral aperture-guiding ring distance increased from left to right, grouping species with a longer stylet and more posterior guiding ring position on the right side (Figure 3.8A-D). According to their position along the y-axis in Figure 3.7A (Factor 2), the length of the female tail (> c′ ratio) increased, and size of lip region (c ratio) decreased from bottom to top along the y-axis. Thus, when projected on the plane of

Factors 1 and 2 in Figure 3.8A (43.3% of the total variance), species with a narrower lip region, longer stylet, longer female tail, and a more posterior guiding ring are located in the right-top quadrant, with a clear distinction of *X. madeirense* specimens from the rest of the species. Species with a wider lip region and shorter female tail are located in the bottom quadrants below y = 0, i.e. *X. plesiopachtaicum* sp. nov. and *X. incertum*, with most specimens of the latter species located in the right-bottom quadrant owing to their longer stylet and more posterior guiding-ring position, except for three specimens that were located in the top quadrant. Of the remaining three species, specimens of *X. parapachydermum* are located in the top-left quadrant because of their short stylet, anterior guiding ring, narrow lip region, and long female tail, but overlap with some specimens of *X. vallense* sp. nov. and *X. pachtaicum* that showed similar values for characters associated with Factor 2. According to their position on the y-axis in Figure 3.7B-D (Factor 3), nematode body length and a ratio increased from the bottom to top. When projected on the plane of Factors 1 and 3 in Figure 3.8B (40.8% of total variance), specimens of *X. vallense* sp. nov. and *X. madeirense* characterized by a longer body and a higher a ratio are located above y = 0, whereas those of *X. pachtaicum* and *X. incertum* with opposite values for these two characters are located below x = 0. The position of the vulva increased and the b ratio decreased from bottom to top along the y-axis in Figure 3.7C (Factor 4), the position of the vulva increased and the b ratio decreased from bottom to top along the y-axis. In Fiugre 3.8C specimens are projected on the plane of Factors 1 and 4 (34.7% of total variance). Although most of the species showed in-between values for those characters associated with Factors 1 and 4 that located most of the specimens around y = 0, x = 0, this factor combination allowed *X. incertum* and *X. madeirense* to be distinguished as two distinct groups owing to their differences in the characters associated with Factors 4 and 1.

Moreover, overall there was wide spatial separation amongst the six *Xiphinema* species when projected on the plane of Factors 2 and 3 (Figure 3.8D), with most of the specimens belonging to a given species located close to each other. Thus, longer nematode species with a higher a ratio are located above y = 0, i.e. *X. madeirense* and *X. vallense* sp. nov., with specimens of *X. madeirense* located in the top-right quadrant because of its longer female tail and narrower lip region and those of *X. vallense* sp. nov.

located in the middle part of the plane and around x = 0. By contrast, shorter nematode species with a lower a ratio are located in the bottom quadrants, with the *Xiphinema* spp. with a shorter female tail and wider lip region located in the bottom-left quadrant, i.e. *X. incertum*, whereas species with a longer female tail and lip region are located in the bottom-right quadrant, i.e. most of the X. pachtaicum specimens. Finally, specimens of *X. plesiopachtaicum* sp. nov., characterized by middle body length and a ratio but long female tail and wide lip region, were located in the left part of the plane and around y = 0. The specimens of the remaining species, *X. parapachydermum*, although located close to each other, overlapped with specimens belonging to some of the other species, particularly with those of *X. pachtaicum* (Figure 3.8D).

MANOVA of the combined morphometric characters data set and *X. americanum*-group species showed significant amongst-species variation (Wilk's lambda = 0.019, F = 16.69, *P* < 0.0001). Eigenvectors of characteristic roots in the MANOVA analysis indicated that c′ ratio and lip region width were the characters with the overall greatest influence, whereas b ratio, V, and tail length showed in-between weights (data not shown). No single morphometric character could be used to discriminate amongst the six species in the study. A set of characters was therefore needed to obtain a degree of separation amongst them. In this respect, the two new species identified in this study showed significant differences (*P* < 0.0001) from the four remaining species within the *X. pachtaicum*-subgroup, although the differences are associated with different characters. Body length, lip region width, and c and c′ ratios contributed the most to differentiating *X. plesiopachtaicum* sp. nov. from the previously described species, whereas stylet length, body length, and hyaline region length can be used to discriminate *X. vallense* sp. nov. from the previous species within the *X. pachtaicum*-subgroup. The differences between these two species were related mostly to body length; and lip region width contributed the most to discriminate between these two new described species (Table 3.8, Figure S3.17). Concerning the previously known species in the *X. pachtaicum*-subgroup, body length, hyaline region length, stylet length, and oral aperture-guiding ring distance are needed to discriminate *X. pachtaicum*; stylet length, female tail length, a, b, and c ratios, hyaline region length, and V for *X. parapachydermum*; c, a, and c′ ratios for *X.*

incertum; and c′ ratio and stylet length for *X. madeirense* (Table 3.8, Figure S3.17).

Table 3.8 Standardized canonical coefficients from Multivariate Analysis of Variance (MANOVA) derived from nematode morphometric characters for selected comparisons of ***Xiphinema plesiopachtaicum* sp. nov. (*X.ples*)**, ***Xiphinema vallense* sp. nov. (*X.vall*)**, one population of *X. madeirense* (*X.made*) two populations of *X. incertum* (*X.ince*) three populations of *X. parapachydermum* (*X.para*), and seven populations of *X. pachtaicum* (*X.pach*)[a].

	Comparison[c]					
Character[b]	***X.ples***	***X.vall***	***X.pach***	***X.para***	***X.ince***	***X.made***
Body length (L)	0.798	-0.530	0.844	-0.173	-0.321	0.201
A	-0.310	-0.382	0.103	0.710	0.515	0.213
B	0.372	-0.232	-0.322	-0.512	0.050	-0.176
C	-0.814	-0.054	0.450	0.533	0.512	0.194
c´	0.562	0.214	0.381	-0.317	1.280	1.021
V	-0.060	-0.267	-0.432	-0.584	0.228	-0.351
Stylet length	0.134	0.798	0.620	0.954	-0.021	0.893
Oral aperture-guiding ring distance	0.381	0.006	-0.597	-0.234	0.324	0.059
Lip region width	-0.700	0.474	0.173	0.044	0.147	0.040
Tail length	-0.168	0.132	0.365	0.868	0.147	0.447
Hyaline region length	0.339	0.507	-0.799	-0.637	0.191	-0.005

[a] Based on 31 female specimens of ***Xiphinema plesiopachtaicum* sp. nov.**, 21 female specimens of ***Xiphinema vallense* sp. nov.**, 9 female specimens of one population of *X. madeirense*, 6 and 7 female specimens of two populations of *X. incertum*, 10, 8, and 7 female specimens of three populations of *X. parapachydermum*, and 10, 9, 9, 10, 10, 6 and 10 female specimens of seven populations of *X. pachtaicum*. All populations from Spain. Standardized canonical coefficients >0.5 are underlined.

[b] Morphological and diagnostic characters according to Lamberti and Ciancio (1993) with some inclusions.

[c] One-single-degree of freedom contrast between the indicated species and the remaining five species in the study.

3.4 Phylogenetic relationships of the *Xiphinema americanum*-group

The amplification of the D2D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial coxI regions yielded single fragments of approximately 800, 1030, and 400 bp, respectively, based on gel electrophoresis. Sequences from other species of the *X. americanum*-group obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) were used for further phylogenetic studies. Sequences for X*. plesiopachtaicum* sp. nov., *X. vallense* sp. nov., *X. astaregiense* sp. nov., *X. luci*, and *X. madeirense* were obtained for these species in this study for the first time. Sequences obtained in this study for *X. brevisicum*, *X. duriense*, *X. incertum*, *X. opisthohysterum*, *X. pachtaicum*, *X. parapachydermum*, and *X. rivesi* matched well with sequences already deposited in GenBank except for one population of *X. rivesi* from Pennsylvania, USA, which extended the diversity of these species to new areas.

Phylogenetic analyses (BI and ML) of the *X. americanum*-group based on a multiple edited alignment of D2-D3 expansion segments of 28S rRNA of a multiple edited alignment including 72 sequences and 762 total characters showed two clearly separated clades, one of them supported (Figure 3.9). Differences in topology were detected for some subclades in clade I using the BI or ML approach (Figure 3.9). The phylogenetic tree resolved two major clades: (I) a well-supported clade [PP = 100%; bootstrap support (BS) = 99%] with 40 sequences including *X. luci* and *X. rivesi* and other 20 species; whereas clade II, which was not well supported [PP = 84%; BS = 49%], contains 31 sequences, including *X. plesiopachtaicum* sp. nov., *X. vallense* sp. nov., *X. astaregiense* sp. nov., *X. brevisicum*, *X. duriense*, *X. incertum*, *X. madeirense*, *X. opisthohysterum*, *X. pachtaicum*, and *X. parapachydermum* amongst others (Figure 3.9). In clade I, *X. luci* (KP268965) clustered with *Xiphinema tarjanense* and *Xiphinema floridae*; and *X. rivesi* (KP268971) clustered with *X. rivesi* from Spain (five sequences), but separated from X. rivesi (AY210845, Proser, USA; and AY601589, Pennsylvania, USA). Although clade II was not well supported, several subclades within it did have good support: (i) *X. pachydermum*, *X. pachtaicum*, *X. plesiopachtaicum* sp. nov. (KP268956–KP268958), *X. astaregiense* sp. nov. (KP268955) *X. parapachydermum*, *X. incertum*, and *X. pachtaicum*; (ii*) X. duriense* with *X.*

opisthohysterum; and (iii) *X. madeirense* with *X. paratenuicutis*. By contrast, *X. simile*, *Xiphinema parasimile*, and *X. vallense* sp. nov. occupied positions in this tree that were not well supported. Finally, *X. brevisicum*, *X. madeirense* (KP268966), *X. duriense* (KP268963), and *X. opisthohysterum* (KP268967) occupied basal positions within this tree that were not well resolved (Figure 3.9).

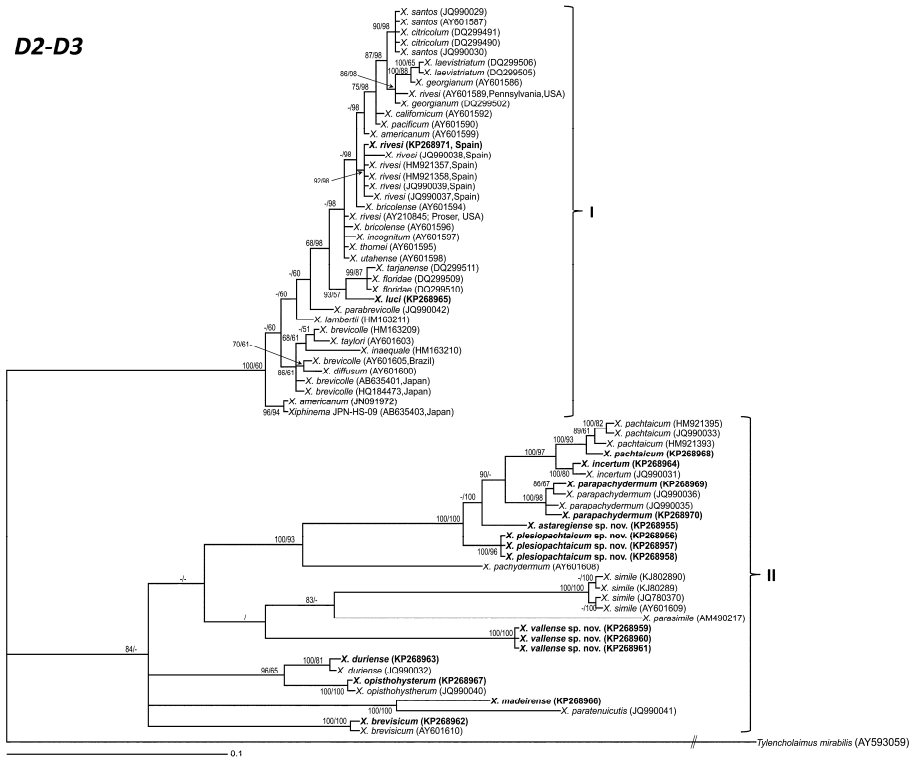

Figura 3.9: Phylogenetic relationships within the *Xiphinema americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from D2-D3 expansion segments of 28S rRNA sequence alignment under the general time reversible model with invariable sites and gamma-shaped distribution. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in the maximum likelihood analysis. Sequences newly obtained in this study are in bold. Scale bar = expected changes per site.

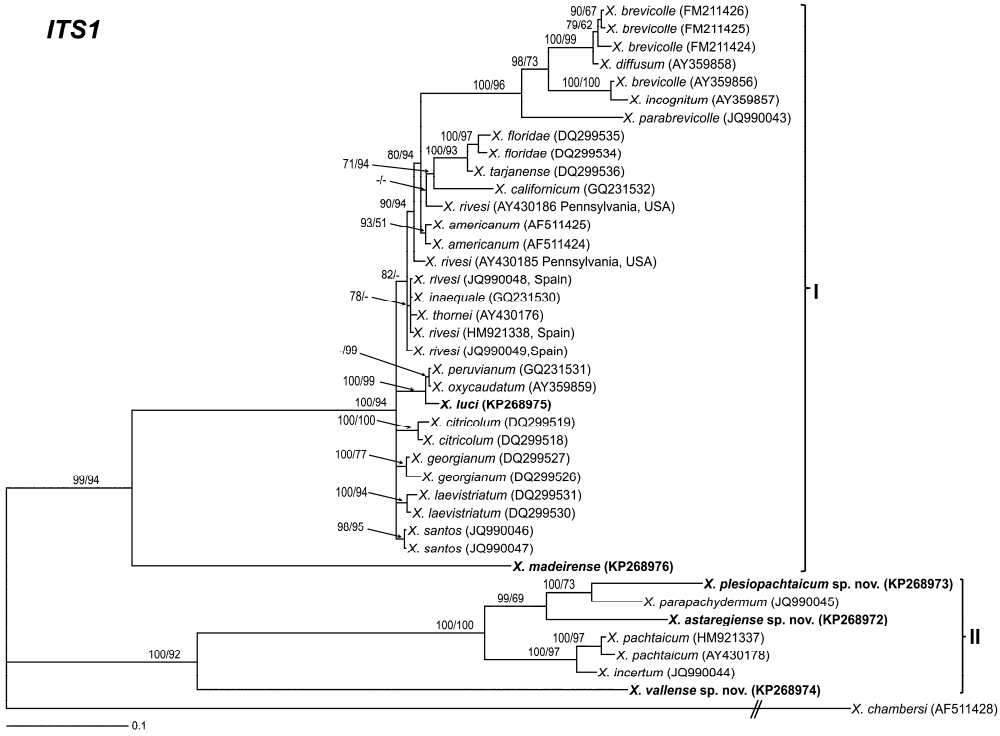

Figura 3.10: Phylogenetic relationships within the *Xiphinema americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from internal transcribed spacer 1 (ITS1) rRNA sequence alignment under the general timereversible and gamma-shaped distribution model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in the maximum likelihood analysis. Sequences newly obtained in this study are in bold. Scale bar = expected changes per site.

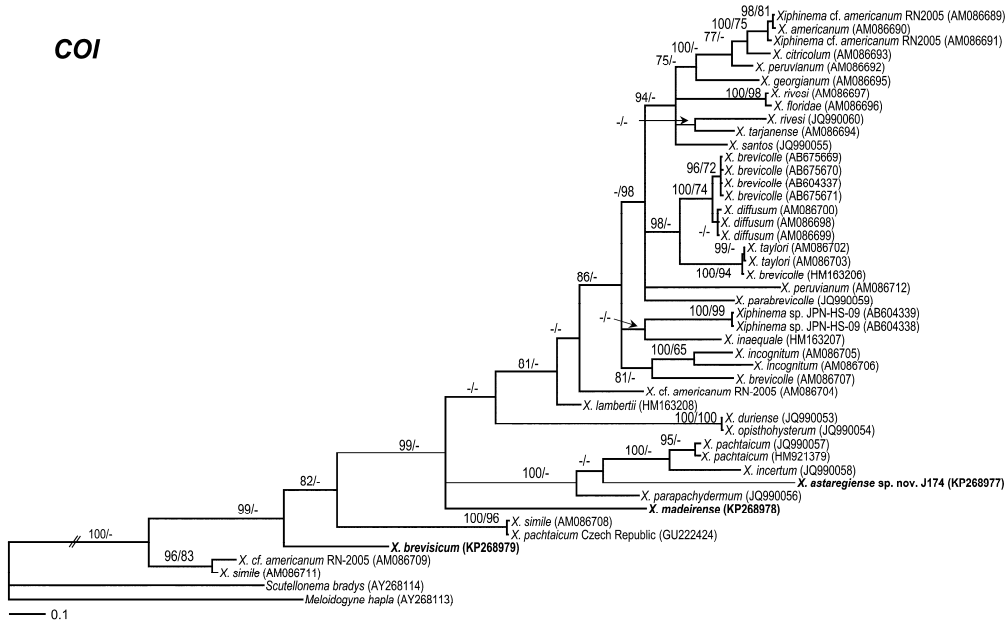

Figura 3.11: Phylogenetic relationships within the *Xiphinema americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from partial cytochrome c oxidase subunit I (coxI) sequence alignment under a transversional of invariable sites and gamma-shaped distribution model TVM + I + G model. Posterior probabilities more than 65% are given for appropriate clades; bootstrap values greater than 50% are given on appropriate clades in the maximum likelihood analysis. Sequences newly obtained in this study in this study are in bold. Scale bar = expected changes per site.

Difficulties were experienced with the alignment of the ITS1 sequences because of scant homology, and only related sequences were included in our study using *Xiphinema chambersi* (AF511428) as the outgroup (Gutiérrez-Gutiérrez et al. 2012). The phylogenetic tree based on ITS1 sequences resolved two clearly separated major clades (Figure 3.10). Clade I, a well-supported clade (PP = 99%; BS = 94%), included 19

species separated in moderately supported clades, and *X. madeirense* (KP268976) in a basal position. Clade II, also well supported (PP = 100%; BS = 92%), included six species separated into the following two subclades: (1) *X. pachtaicum* (HM924337, AY430178), *X. incertum* (JQ990044), *X. astaregiense* sp. nov. (KP268972), *X. plesiopachtaicum* sp. nov. (KP268973), and *X. parapachydermum* (JQ990045); and (2) *X. vallense* sp. nov. (KP268974).

Phylogenetic analyses (BI and ML) of the *X. americanum*-group based on the partial coxI gene of a multiple edited alignment including 46 sequences with 405 positions in length showed several clades with low or no support with the exception of some species relationships deep in the phylogenetic tree (Figure 3.11). Our sequences of *X. astaregiense* sp. nov. and *X. madeirense* clustered in the *X. pachtaicum*-subgroup (including *X. pachtaicum*, *X. incertum*, *X. parapachydermum*), whereas *X. brevisicum* clustered clearly separated in a basal position of the tree (Figure 3.11).

Morphological characters mapping showed a feasible ancestral stage for vulva position, a ratio, and stylet length (Figure 3.12). Vulva position seems to be evolved from an anterior position to a more posterior position in the tree (Figure 3.12). The *X. pachtaicum*-subgroup (clade II) was characterized by a higher V value in the species group studied in the present research. However, there are some exceptions, such as *Xiphinema paratenuicitus*, which has a more anterior vulva position (Figure 3.12). SIMMAP and MESQUITE analyses showed that in their ancestral stages *X. americanum*-group nematodes were longer with a similar maximum body width (*viz. X. brevicolle*, *X. georgianum*, *X. inaequale*, *X. taylori*). These characters were associated with the *X. pachtaicum*-subgroup (clade II). Some exceptions were found between the two subgroups, *X. americanum* 'sensu stricto' subgroup (clade I) and *X. pachtaicum*-subgroup (clade II). These exceptions were *X. pacificum*, *X. utahense*, *X. incertum* and *X. brevisicum* (Figure 3.12). On the contrary, the stylet length in this group seems to have evolved from a shorter to a longer stylet. In this case, reversal(s) may have occurred in some species (e.g. *X. tarjanense*, *X. americanum*, *Xiphinema citricolum*, *Xiphinema laevistriatum*, *Xiphinema thornei*, and *Xiphinema diffusum*).

4. Discussion

The primary objective of this study was to identify and molecularly characterize species belonging to the *X. americanum*-group associated with cultivated and wild olives in southern Spain. Our results demonstrate that the use of multivariate analyses applied for morphological studies integrated with rDNA and mtDNA molecular markers deciphered diversity in this difficult group of nematodes and particularly in the *X. pachtaicum*-species complex. We have described here three new species of the *X. americanum*-group based upon integrative taxonomy and the phylogenetic relationships amongst the new and known species of this group based upon nuclear rDNA and mtDNA.

4.1 Morphological comparison of studies *Xiphinema plesiopachtaicum* sp. nov., *Xiphinema vallense* sp. nov., *Xiphinema astaregiense* sp. nov. with related taxa.

Using the polytomous key of Lamberti *et al.* (2004) and sorting on features A (odontostyle length), B (vulva position), and H (tail end shape), *X. plesiopachtaicum* sp. nov. groups with *X. pachtaicum*, *X. parapachydermum* Gutiérrez-Gutiérrez *et al.* 2012, and *X. vallense* sp. nov. On the one hand, the morphology and morphometrics of *X. plesiopachtaicum* sp. nov. agree closely with the original and other populations of *X. pachtaicum* from Italy described by Lamberti and Siddiqi (1977), except for small differences in the c and c′ ratios (Table 3.9). By contrast, *X. plesiopachtaicum* sp. nov. differs from paratypes of *X. vallense* sp. nov. by small differences in the a ratio and the absence of males. In addition, the new species showed some small differences from other species in this group in measurements and ratios, including body length, a, c, c′, V, lip region width, and tail hyaline region (Table 3.9).

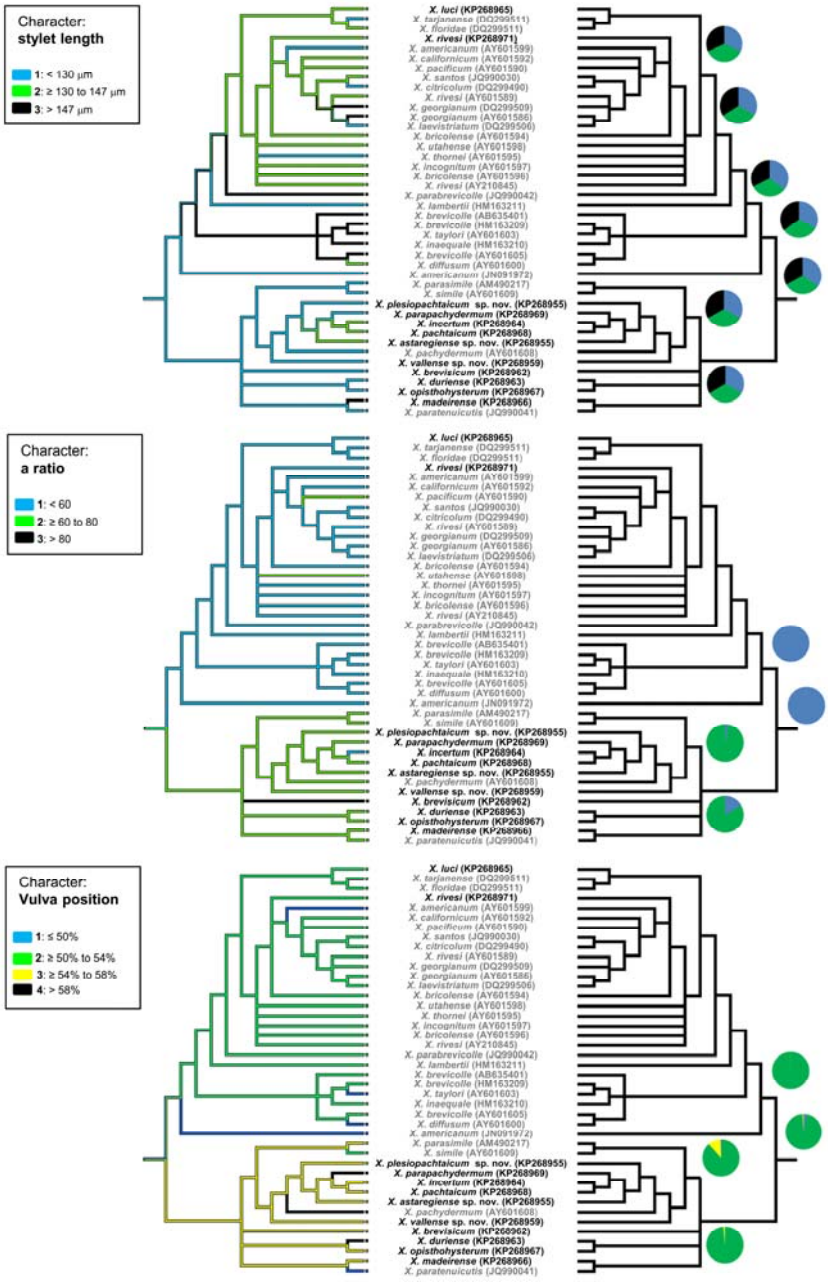
Character:
stylet length
1: < 130 µm
2: ≥ 130 to 147 µm
3: > 147 µm
Character:
a ratio
1: < 60
2: ≥ 60 to 80
3: > 80
Character:
Vulva position
1: ≤ 50%
2: ≥ 50% to 54%
3: ≥ 54% to 58%
4: > 58%
X. luci (KP268965)
X. tarjanense (DQ299511)
X. floridae (DQ299511)
X. rivesi (KP268971)
X. americanum (AY601599)
X. californicum (AY601592)
X. pacificum (AY601590)
X. santos (JQ990030)
X. citricolum (DQ299490)
X. rivesi (AY601589)
X. georgianum (DQ299509)
X. georgianum (AY601586)
X. laevistriatum (DQ299506)
X. bricolense (AY601594)
X. utahense (AY601598)
X. thornei (AY601595)
X. incognitum (AY601597)
X. bricolense (AY601596)
X. rivesi (AY210845)
X. parabrevicolle (JQ990042)
X. lambertii (HM163211)
X. brevicolle (AB635401)
X. brevicolle (HM163209)
X. taylori (AY601603)
X. inaequale (HM163210)
X. brevicolle (AY601605)
X. diffusum (AY601600)
X. americanum (JN091972)
X. parasimile (AM490217)
X. simile (AY601609)
X. plesiopachtaicum sp. nov. (KP268955)
X. parapachydermum (KP268969)
X. incertum (KP268964)
X. pachtaicum (KP268968)
X. astaregiense sp. nov. (KP268955)
X. pachydermum (AY601608)
X. vallense sp. nov. (KP268959)
X. brevisicum (KP268962)
X. duriense (KP268963)
X. opisthohysterum (KP268967)
X. madeirense (KP268966)
X. paratenuicutis (JQ990041)

Figura 3.12: Morphological character history reconstruction for three morphometric characters using Bayesian simulations (trees on right) and parsimony (trees on left) on the D2-D3 consensus tree using only one or few sequences for each Xiphinema species. Charts on selected nodes show relative posterior probabilities of each stage of the character. (A) stylet length, 1: < 130 µm, 2: ≥ 130–147 µm, 3: > 147 µm. (B) body length/maximum body width (a), 1: < 60, 2: 61–80 and 3: > 80. (C) distance from anterior end to vulva as percentage of body length (V; %), 1: ≤50%, 2: 51–54%, 3: 55–58% and 4: > 58%.

Table 3.9 Differential morphometrics (minimum and maximum values) of ***Xiphinema plesiopachtaicum* sp. nov.**, ***Xiphinema vallense* sp. nov.**, and ***Xiphinema astaregiense* sp. nov.** from paratypes of *Xiphinema americanum*-group species complex. (All measurements in µm).

Chraracters/Ratio	***plesiopachtaicum* sp. nov.**	*parapachydermum*	***vallense* sp. nov.**	*pachtaicum**
L	1520-2078	1411-2000	1830-2228	1500-2100
a	57.3-70.2	51.3-73.1	61.6-79.1	47-71
c	62.5-88.7	46.3-75.5	58.2-86.3	47-76
c'	1.3-1.7	1.5-2.3	1.4-1.7	1.5-2.1
V%	55.5-60.0	55-66	55.0-59.5	53-60
Odontostyle length	77.0-89.0	70.0-87.5	73.0-85.5	77-102
Lip region width	8.5-10.5	8.0-9.5	8.0-9.0	-
J	5.5-10.0	7.0-12.5	6.5-8.5	-
Male	not found	frequent	extremely rare	extremely rare
	***vallense* sp. nov.**	*parapachydermum*	***plesiopachtaicum* sp. nov.**	*pachtaicum**
L	1830-2228	1411-2000	1520-2078	1500-2100
a	61.6-79.1	51.3-73.1	57.3-70.2	47-71
c	58.2-86.3	46.3-75.5	62.5-88.7	47-76
c'	1.4-1.7	1.5-2.3	1.3-1.7	1.5-2.1
V%	55.0-59.5	55-66	55.5-60.0	53-60
Odontostyle length	73.0-85.5	70.0-87.5	77.0-89.0	77-102
Lip region width	8.0-9.0	8.0-9.5	8.5-10.5	-
J	6.5-8.5	7.0-12.5	5.5-10.0	-
Male	extremely rare	frequent	not found	extremely rare
	***astaregiense* sp. nov.**	*diffusum*	*rivesi*	*incognitum*
L	2740-3018	1600-1800	1680-2110	1700-2100
a	66.8-76.1	46-51	37-49	41-49
c	112.2-129.9	63-84	51-59	47-75
c'	0.9-1.1	0.8-1.1	1.3-1.5	0.9-1.3
V%	55.5-59.0	47-52	51-54	48-53
Odontostyle length	82.0-89.0	84-89	90-101	82-93
Lip region width	8.5-10.5	10.0-12.0	10.0-11.0	11.0-13.0
J	7.5-9.0	10.0.14.0	-	8.5-12.5
Male	frequent	not found	extremely rare	extremely rare

[a] Measurements are in µm and in the form: mean ± standard deviation (range).

[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

*After Lamberti and Siddiqi (1977)

Similarly, based upon other diagnostic characters in the *X. americanum*-group viz. A, B, C (c′ ratio), and G (lip region) (Lamberti et al. 2004), *X. vallense* sp. nov. groups with *X. pachtaicum*, *X. parapachydermum*, and *X. plesiopachtaicum* sp. nov. *Xiphinema vallense* sp. nov. differs from paratypes of *X. plesiopachtaicum* sp. nov. by small differences in the a ratio and the presence of males (Table 3.9). In addition, *X. vallense* sp. nov. shows some small differences from other species in this group in measurements and ratios, including body length, a, c, c′, V, lip region width, tail hyaline region, and frequency or presence/absence of males (Table 3.9).

Finally, using the polytomous key of Lamberti et al. (2004) and sorting on features A, C, and H, *X. astaregiense* sp. nov. groups with *X. diffusum* Lamberti and Bleve-Zacheo 1979, *X. incognitum* Lamberti and Bleve-Zacheo 1979, and *X. rivesi* Dalmasso 1969. *Xiphinema astaregiense* sp. nov. shows some differences from the paratypes of these species in measurements and ratios, including body length, a, c, c′, V, lip region width, tail hyaline region, and frequency or presence/absence of males (Table 3.9).

4.2 Comparative multivariate analysis of morphometric characters of *Xiphinema plesiopachtaicum* sp. nov., *Xiphinema vallense* sp. nov., *Xiphinema astaregiense* sp. nov., and *Xiphinema pachtaicum*-subgroup species.

Multivariate analyses, including principal coordinates, hierarchical cluster, and canonical discriminant analyses, have proven to be useful tools for species delimitation within the genus *Xiphinema* (Cho and Robbins 1991, Roca and Bravo 1997, Gutiérrez-Gutiérrez *et al.* 2013). Lamberti and Ciancio (1993) and subsequently Lamberti *et al.* (2002) analysed the species diversity of the *X. americanum*-group using a hierarchical cluster analysis of morphometrics. Other multivariate analyses, such as factor analysis or principal components analysis (PCA), also enable to compare taxonomically similar species (Legendre and Legendre 1998, Palomares-Rius *et al.* 2010, Cantalapiedra-Navarrete *et al.* 2013). Factor analysis or PCA has also been used to resolve the taxonomy of diverse groups of

organisms, including vascular plants (Borba *et al.* 2002, Kuta *et al.* 2014), invertebrates (Vďačný *et al.* 2014), and vertebrates (Bärmann *et al.* 2013).

Morphological identification of *X. americanum*-group species is problematic. Moreover, the rising number of species in this group has increased the difficulty of new species diagnosis because of the close relationships amongst them. In this regard, the use of a single or few characters is generally insufficient to characterize a species because of wide intra-population and intraspecific variation. Therefore, multivariate analyses appear to be useful for species diagnosis as well as to define the relationships amongst species or groups of species. By hierarchical cluster analysis based on seven morphometric characters, Lamberti and Ciancio (1993, 1994) established five subgroups within the X*. americanum*-group: the *Xiphinema brevicolle*-, *X. americanum*-, *Xiphinema taylori*-, *X. pachtaicum*-, and *Xiphinema lambertii*-subgroups. The *X. pachtaicum*-subgroup, composed of eight species (*X. fortuitum*, *X. incertum*, *X. madeirense*, *X. opisthohysterum*, *X. pachtaicum*, *X. pachydermum*, *X. simile*, and *X. utahense*) was characterized by nematode species with a body length of c. 2 mm, lip region set off from body profile, V about 55%, b ratio of c. 6.8, total stylet length of c. 133 µm, and tail with a pointed terminus.

The results of the multivariate analyses identified body length, a, b, c and c′ ratios, V, total stylet length, and lip region width as key morphometric characters to differentiate a restricted set of species within the *X. pachtaicum*-subgroup in the study (*X. incertum*, *X. madeirense*, *X. pachtaicum*, *X. parapachydermum*, *X. plesiopachtaicum* sp. nov., and *X. vallense* sp. nov.; Table 3.7). Indeed, although some specimens for a given species show values outside the overall mean value of the species for some morphometric characters, making it difficult to identify a given specimen to species level based only on morphometric characters, in our study some of the species could be differentiated using a discrete number of characters (Figure 3.7, 3.8). Thus, based on multivariate factor analyses *X. madeirense* and *X. incertum* specimens form two clearly distinct groups, but the contrary occurs for *X. pachtaicum* and *X. parapachydermum*, which share similar values for most of the characters included in the study. The remaining species form a more compact group with a lesser degree of

variation in the morphometric characters compared with that observed amongst the *X. pachtaicum*-subgroup. Consequently, *X. plesiopachtaicum* sp. nov. and *X. vallense* sp. nov. could be integrated into the *X. pachtaicum*-subgroup species, despite not fulfilling the diagnostic parameters (see below) for this species subgroup established by Lamberti and Ciancio (1993).

The results of the MANOVAs emphasize the differences in morphometric characters amongst the *X. pachtaicum*-subgroup species (Table 3.8). Indeed, to some degree all of the species could be discriminated based on a set of morphometric characters. Thus, *X. madeirense* clearly differs from the rest of species based upon stylet length and c′ ratio, agreeing with the results of the factor analysis (Table 3.7), and these species are closely related to feeding apparatus and female tail shape (Jairajpuri and Ahmad 1992). However, *X. incertum* did not show clear differences except for those in a, c, and c′ ratios (Figure S3.17). By contrast, vulva position showed a constant value amongst the majority of the species (Figure S3.17), which confirms this character as a discriminant feature for *X. pachtaicum*-subgroup species as described by Lamberti and Ciancio (1993, 1994), and as an important taxonomic character for nematode diagnostics (Jairajpuri and Ahmad 1992).

Xiphinema plesiopachtaicum sp. nov. and *X. vallense* sp. nov. share similar morphology in diagnostic characters including vulva position, oral aperture-guiding distance, and hyaline region length. However, variation in body length and lip region width can be used to discriminate between them (Table 3.8, Figure S3.17). Both species are also close to *X. parapachydermum* and *X. pachtaicum* in general morphology (Table 3.9). Morphometrically, *X. plesiopachtaicum* sp. nov. can be distinguished from these species by body length, hyaline region length, and a, b, c, and c′ ratios (Figure S3.17), and also from the remaining *X. pachtaicum*-subgroup species studied owing to a wider lip region and shorter female tail (Figure S3.17). *Xiphinema vallense* sp. nov. can be differentiated by body length, hyaline region length, and a and c ratios from X. pachtaicum and *X. parapachydermum* (Figure S3.17) and particularly by stylet length, which can be used as a discriminant morphometric character for this species.

Finally, based on the morphometric multivariate analyses as well as morphological diagnostic characters, *X. plesiopachtaicum* sp. nov. and *X. vallense* sp. nov. are here included in the *X. pachtaicum*-subgroup. The differences between these two species are related mostly to body length and lip region, as well as molecular markers. However, both taxa are cryptic to human perception largely because of the lack of conspicuous differences in morphometric and morphological appearance (Palomares-Rius *et al.* 2014) and therefore a set of several characters measured in numerous specimens are needed to discriminate morphometrically amongst them.

4.3 Molecular and phylogenetic relationships in the *Xiphinema americanum*-group.

Sequences of nuclear rDNA and mtDNA genes, particularly D2-D3, ITS1, and partial coxI, have proven to be a powerful tool for providing accurate and molecular species identification in Longidoridae (Chen *et al.* 2005, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2012, 2013, Zasada *et al.* 2014). Our results confirm the usefulness of these markers in the *X. americanum*-group, as nucleotide differences amongst species ranged from 26 to 126 nucleotides for D2-D3, from eight to 270 nucleotides for ITS1, and from seven to 108 nucleotides for partial coxI within related sequences. The phylogenetic relationships inferred in this study based on the D2-D3 and ITS1 sequences mostly agree with the lineages obtained by Gutiérrez-Gutiérrez *et al.* (2010, 2012) and Zasada *et al.* (2014) for the phylogeny of the *X. americanum*-group.

In order to understand the evolution of the *X. americanum*-group, it is important to confirm a correlation between the results obtained by conventional morphological approaches and new molecular methods. Two clearly separated major subgroups (clade I and II) were shown using both nuclear rDNA molecular markers (D2-D3 and ITS1). One subgroup was formed by *X. americanum* 'sensu stricto' (clade I), whereas the other group was formed by other species (clade II; Figure 3.9, 3.10). Species from clade II are more variable morphologically and morphometrically than the subgroup *X. americanum* 'sensu stricto', as showed the long- branch in the phylogenetic tree. The phylogeny separation for clade I was observed in

coxI; however, it was not supported, probably because of its high mutation rate and the smaller fragment used in this study. However, coxI is a good marker for molecular identification in *X. americanum*-group as bar-coding. The three new species (*X. astaregiense* sp. nov., *X. plesiopachtaicum* sp. nov., and *X. vallense* sp. nov.) were studied phylogenetically here and we have provided new sequences for *X. madeirense* and *X. luci* that may help with their identification (D2-D3 and ITS1, and coxI for the latter). The new sequence of *X. rivesi* provided in this research nested within the clade containing all previously sequenced Spanish populations. These sequences were different to other *X. rivesi* sequences deposited in GenBank, indicating the possibility of cryptic speciation as suggested by Gutiérrez-Gutiérrez *et al*. (2012). While for *X. luci* we provide two molecular markers and its phylogenetic position is defined in the genus. This species is closely related phylogenetically to *X. floridae* and *X. tarjanense* using D2-D3, but differs from *X. floridae* by a smaller c′ and a pointed tail tip. It differs from *X. tarjanense* by a smaller odontostyle, higher c′, smaller c, smaller body length, lip region set off from body profile, tail tip with pointed terminus, and longer tail length. *Xiphinema astaregiense* sp. nov. and *X. plesiopachtaicum* sp. nov. were clustered together using D2-D3 and ITS1 markers, whereas the phylogenetic relationship of *X. vallense* sp. nov. with these species was weak. The *X. pachtaicum*-subgroup (clade II) is not well supported in major clades, and some small clades (*viz. X. duriense-X. opisthohysterum*, *X. madeirense–X. paratenuicutis*) and species (*X. brevisicum*) formed polytomies in this subgroup with the D2-D3 marker. *Xiphinema madeirense* and *X. astaregiense* sp. nov. did not show a position agreement with their morphological grouping using the ITS1 marker. Clades I and II showed different evolution rates based on their branch lengths and this could be a major point in resolving some clades. Another possibility is the polyphyletic origin of *X. americanum*-group species and the loss of some species in the clades or the incomplete sampling of this group within the genus *Xiphinema*. The partial coxI phylogeny did not show a clear relationship within the species in the *X. americanum*-group; however, this marker could be used as a good barcoding region in order to identify species.

Mapping morphological character evolution on the tree showed some putative evolution patterns for vulva position, a ratio, and total stylet length.

Vulva position seems to have evolved from an anterior position to a more posterior position in clade II. The *X. pachtaicum*-subgroup (clade II) is characterized by a higher V value in the species groups analysed. This shift in the position is difficult to explain because of the lesser range in vulval position for the -group (clade I) and the short and undifferentiated reproductive *X. americanum* system in comparison to the *X.* non-*americanum*-group species. A more anterior vulva position seems to be more related to the functional regression of the anterior branch (Coomans *et al.* 2001). However, *X. americanum*-group species have both branches functional and equally developed. Ratio a is related to nematode size and maximum body width; SIMMAP and MESQUITE analyses showed that in their ancestral stages nematodes of the *X. americanum*-group species were longer with a similar maximum body width. This character is represented by the *X. pachtaicum*-subgroup (clade II). We can assume this because maximum body width could be a more restricted character in comparison to body length because may be closely related with the soil particles were nematode move. In the MESQUITE analysis, stylet length seems to have evolved in *X. americanum*-group from a shorter to a longer stylet. However, the ancestral stage for the majority of the species was not clear. Nevertheless, stylet length is an important taxonomic and biological character, which is closely related to the feeding apparatus (Jairajpuri and Ahmad 1992). The several reversal states in some species could be related to additional adaptation and selection of specimens with a longer stylet because of vegetation (host-plant) changes during their evolution. Nematodes with a long stylet or semiendoparasitic feeding behaviour can feed on higher quality tissues such as phloematic cells (Wyss 1981, Bockenhoff *et al.* 1996). Mapping characters in Nematoda could be difficult, mainly because fast evolution and the possible restriction of morphotypes for soil habitat. Additionally, the value of individual characters is particularly amplified in organisms with limited cell counts and structural complexity, such as small invertebrates, which comprise the majority of metazoan phyla (Ragsdale and Baldwin 2010). The limited size of nematodes makes complete, three-dimensional reconstruction of entire organ systems a feasible goal (Ragsdale and Baldwin 2010). Characters such as amphidial fovea, tails, and male and female reproductive systems in *Xiphinema* could be investigated using these techniques. In this case, the existence of two clades without any species linking them makes it very difficult to understand

the species evolution and it will be necessary in the future to find species linking both clades in the *X. americanum*-group. *Xiphinema americanum* s.l. species are markedly similar morphologically and probably evolving very fast between them. Which factors contribute to this speciation is still a matter of debate and the occurrence of novel verrucomicrobial species, endosymbiotic and associated with parthenogenesis in *Xiphinema americanum*-group species could help to find an explanation (Vandekerckhove *et al.* 2000). However, more studies are necessary in order to understand the 'possibly' complex relationships of these endosymbiotic bacteria with their hosts and how these shape the phylogeny of this species-group.

5. Conclusions

In summary, the present study has demonstrated the importance of using integrative taxonomic identification highlighting the time-consuming aspect and difficulty of correct identification at species level within the *X. americanum*-group. The study has also provided molecular markers for precise and unequivocal diagnosis of some species of the *X. americanum*-group, which will allow the differentiation of virus vector or quarantine species. This and previous studies have demonstrated that the *X. americanum*-group is clearly a complex group and much work remains to be carried out to elucidate species boundaries in this group of plant-parasitic nematodes. The nematodes of this group are economically important because they vector nepoviruses that cause considerable damage to a variety of agricultural crops.

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8. Suplementary information

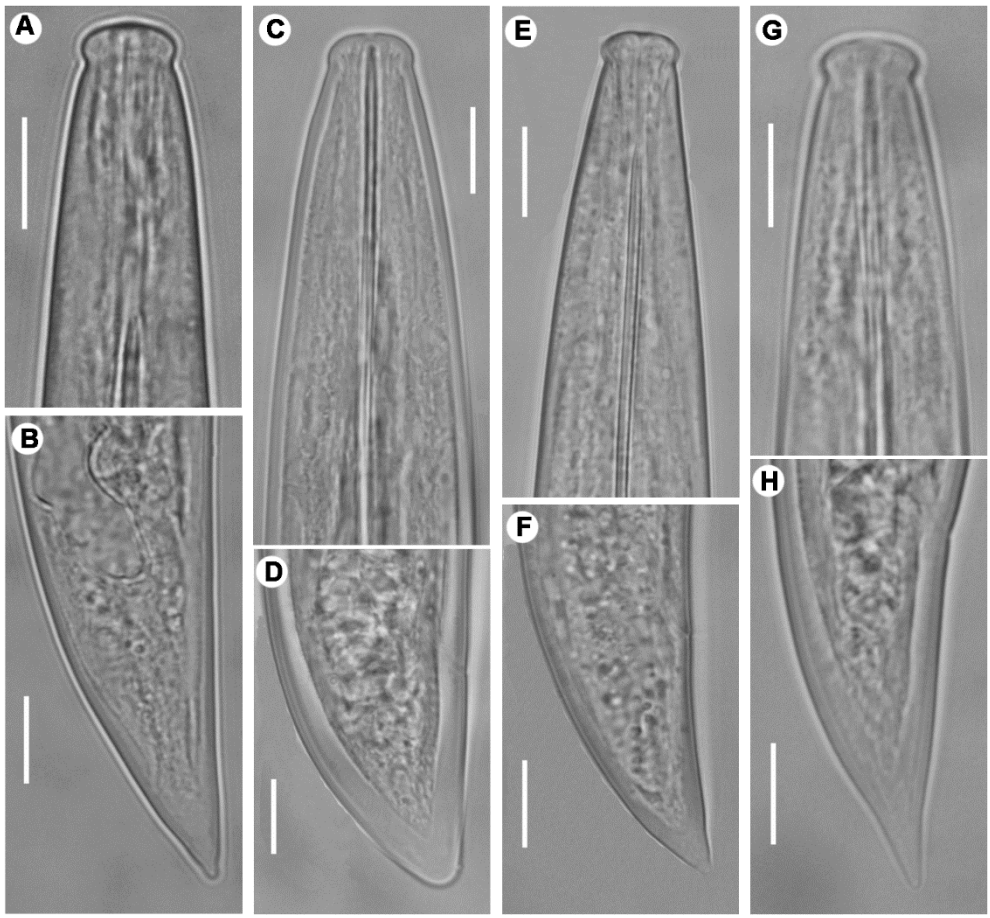

Figura S3.13: Light micrographs of *Xiphinema duriense* Lamberti *et al.* 1993 (A, B), *Xiphinema incertum* Lamberti *et al*. 1983 (C, D), *Xiphinema opisthohysterum* Siddiqi, 1961 (E, F), and *Xiphinema parapachydermum* Gutiérrez-Gutiérrez *et al*. 2012 (G, H) from southern Spain. A, C, E, G, female lip regions; B, D, F, H, female tail regions. Abbreviation: a, anus. Scale bars: A–H = 20 µm.

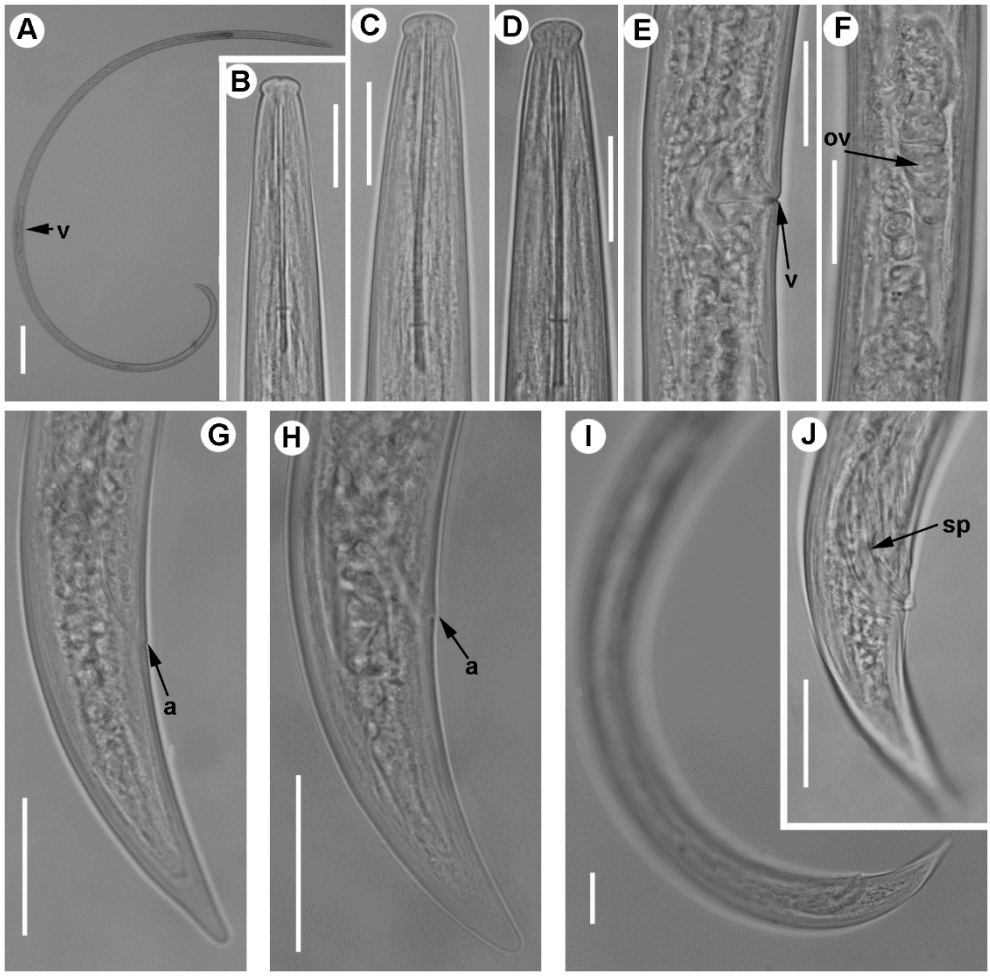

Figura S3.14: Light micrographs of *Xiphinema brevisicum* Lamberti *et al.* 1994, from southern Spain. A, whole female. B–D, female neck regions. E, vulval region. F, detail of ovary. G, H, female tail regions. I, J, male tail. Abbreviations: a, anus; ov, ovary; sp, spicules; V, vulva. Scale bars: A = 100 µm; B–J = 10 µm.

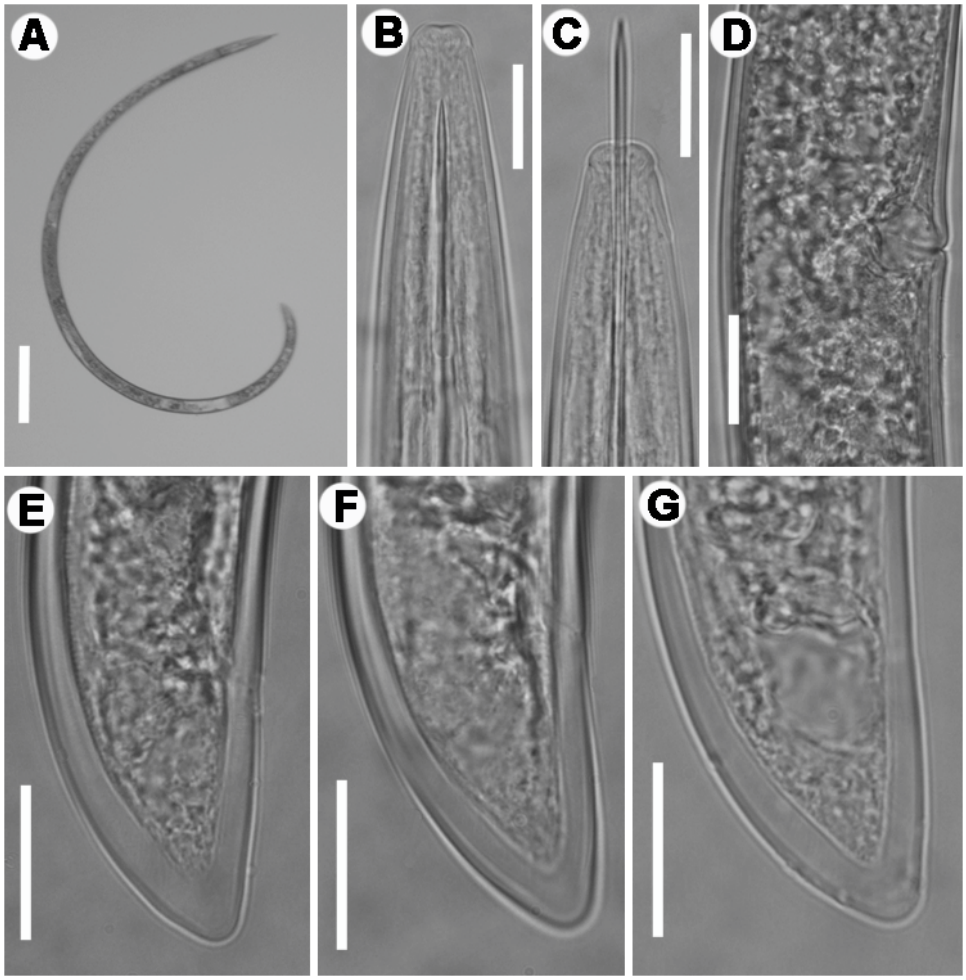

Figura S3.15: Light micrographs of *Xiphinema luci* Lamberti and Bleve-Zacheo 1979, from southern Spain. A, whole female. B, C, female lip regions. D, vulval region. E–G, female tail regions. Scale bars: A = 200 µm; B–G = 20 µm.

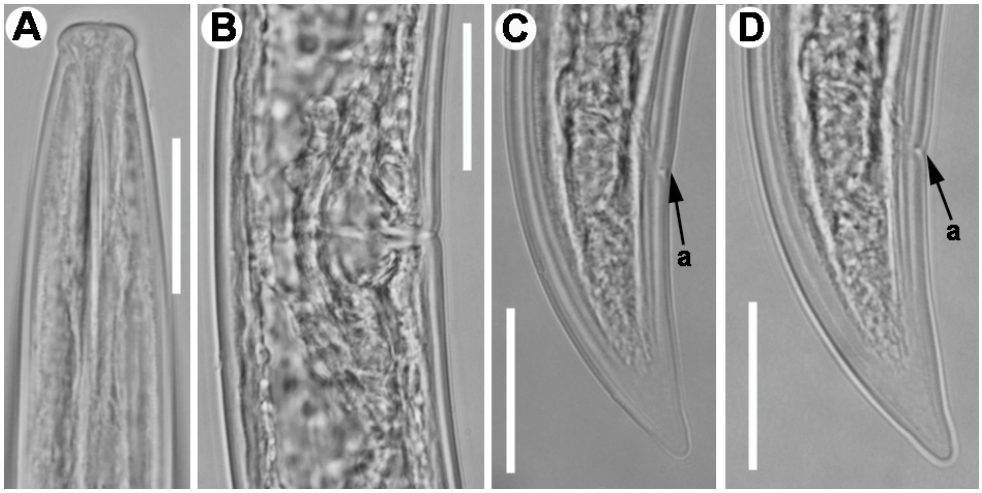

Figura S3.16: Light micrographs of *Xiphinema madeirense* Lamberti *et al*. 1994, from southern Spain. A, female lip region. B, vulval region. C, D, female tail regions. Abbreviation: a, anus. Scale bars = 20 µm.

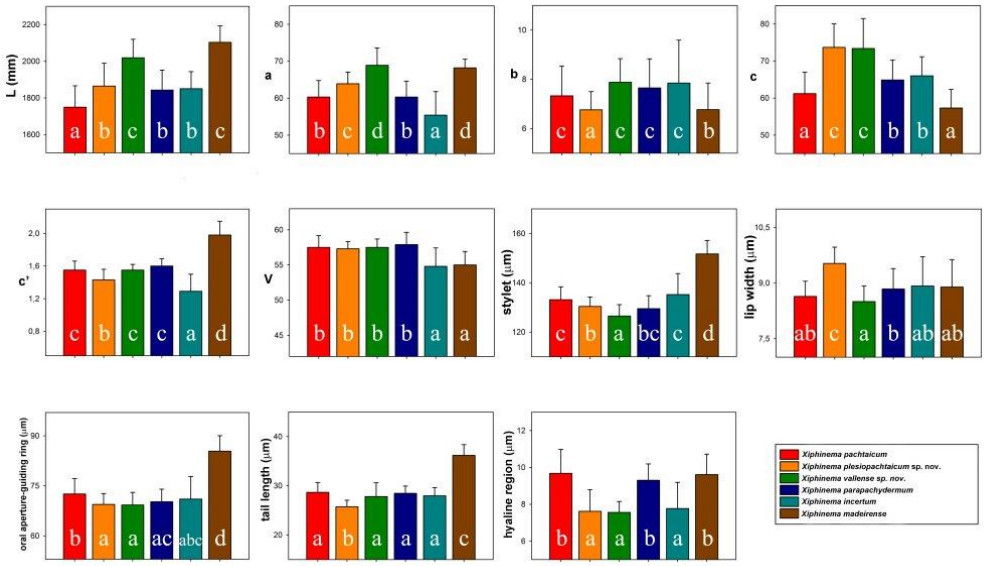

Figura S3.17: Plots of morphometric characters for the six *Xiphinema* spp. in the multivariate analyses in this study. Each bar is the mean of several specimens sampled in Spain as follows: 31 female specimens of ***Xiphinema plesiopachtaicum* sp. nov.**; 21 female specimens of ***Xiphinema vallense* sp. nov.**; nine female specimens of one population of *Xiphinema madeirense*; six and seven female specimens of two populations of *Xiphinema incertum*; ten, eight, and seven female specimens of three populations of *Xiphinema parapachydermum*; and ten, nine, nine, ten, ten, six, and ten female specimens of seven populations of *Xiphinema pachtaicum*. Error bars indicate the SD of the mean. Morphological and diagnostic characters according to Lamberti and Ciancio (1993) with some additions.

Table S3.10 Morphometrics of *Xiphinema brevisicum* Lamberti *et al*. 1994, *Xiphinema duriense* Lamberti *et al*. 1993, *Xiphinema incertum* Lamberti, Choleva and Agostinelli 1983, *Xiphinema luci* Lamberti and Bleve-Zacheo 1979, *Xiphinema madeirense* Lamberti *et al*. 1994, and *Xiphinema opisthohysterum* Siddiqi 1961 from several localities in Spain. All measurements in µm and in the format: mean ± SD (range)*.

Character/life-stage	*X. brevisicum*		*X. duriense*	*X. incertum*	*X. luci*	*X. madeirense*	*X. opisthohysterum*
	Females	**Male**	**Females**	**Females**	**Females**	**Females**	**Females**
n	3	1	3	6	3	9	3
L	2017 ± 172 (1822-2150)	2017	1309 ± 82 (1239-1400)	1854 ± 134 (1683-2016)	1911 ± 189 (1778-2128)	2103 ± 90 (1900-2200)	1559 ± 86 (1505-1658)
a	79.5 ± 3.4 (75.9-82.7)	84.0	51.4 ± 3.2 (48.6-54.9)	59.9 ± 4.7 (52.3-64.7)	48.5 ± 1.9 (46.9-50.7)	68.3 ± 2.3 (64.0-72.0)	48.4 ± 3.3 (46.1-52.2)
b	8.4 ± 0.9 (7.9-9.4)	7.7	5.5 ± 0.3 (5.2-5.7)	6.5 ± 0.4 (6.1-7.3)	9.5 ± 1.0 (8.7-10.6)	6.8 ± 1.1 (5.6-8.8)	6.1 ± 0.8 (5.3-6.9)
c	47.8 ± 5.7 (42.4-53.8)	46.9	45.4 ± 5.8 (39.3-50.0)	66.5 ± 7.1 (60.1-79.1)	65.1 ± 3.9 (60.9-68.6)	57.3 ± 5.0 (48.0-62.0)	57.1 ± 4.3 (52.8-61.4)
c′	2.6 ± 0.2 (2.4-2.8)	2.3	1.9 ± 0.1 (1.8-1.9)	1.4 ± 0.1 (1.3-1.6)	1.1 ± 0.02 (1.1-1.2)	2.0 ± 0.2 (1.8-2.2)	1.6 ± 0.1 (1.4-1.7)
V or T	53.7 ± 1.5 (52.0-55.0)	42.7	65.5 ± 1.3 (64.5-67.0)	57.1 ± 1.4 (55.0-59.0)	51.0 ± 0.0 (51.0-51.0)	55.0 ± 1.9 (52.0-58.0)	63.5 ± 1.8 (62.0-65.5)
G_1	9.2 ± 1.2 (8.0-10.2)	-	6.3 ± 0.3 (6.1-6.2)	-	17.4 ± 0.7 (16.9-17.9)	-	-
G_2	8.3 ± 0.6 (7.6-8.7)	-	6.8 ± 0.5 (6.3-7.2)	-	18.5 ± 1.1 (17.8-19.3)	-	-
Odontostyle length	61.2 ± 0.8 (60.5-62.0)	59	68.3 ± 1.6 (66.5-69.5)	82 ± 2.4 (79-85)	91 ± 3.5 (89-95)	99.9 ± 4.5 (92.5-105.0)	62.7 ± 2.5 (61.0-65.5)
Odontophore length	42 ± 2.6 (39-44)	42	36.8 ± 2.8 (34.5-40.0)	47.8 ± 3.5 (43.0-52.5)	49.7 ± 4.6 (47-55)	50.7 ± 2.3 (47-54)	39.2 ± 1.5 (37.5-40.5)
Lip region width	9.5 ± 0.5 (9.0-10.0)	10.0	8.0 ± 0.3 (7.5-8.0)	8.3 ± 0.5 (7.5-8.5)	10.5 ± 0.3 (10.5-11.0)	9.0 ± 0.7 (8.0-10.0)	9.0 ± 0.8 (8.0-9.5)
Oral aperture-guiding ring	55.3 ± 0.6 (55-56)	54	57.7 ± 0.6 (57-58)	66.8 ± 4.0 (60.5-72.0)	64.3 ± 13.5 (51-78)	85.4 ± 4.6 (76-92)	54.5 ± 1.8 (53.0-56.5)
Tail length	42.3 ± 2.1 (40-44)	43	29 ± 2.2 (27.5-31.5)	28 ± 1.8 (25.5-31.0)	29.3 ± 2.1 (27-31)	36.2 ± 3.3 (34-40)	27.3 ± 1.0 (26.6-28.5)
J	9.0 ± 0.5 (8.5-9.5)	10.0	7.0 ± 1.0 (6.0-8.0)	8.8 ± 1.0 (7.5-10.0)	7.5 ± 0.5 (7.0-8.0)	9.5 ± 1.1 (8.0-12.0)	7.5 ± 1.3 (6.5-9.0)
Spicules	-	35	-	-	-	-	-
Lateral accessory piece	-	8.0	-	-	-	-	-

[a] Measurements are in µm and in the form: mean ± SD (range).

[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

Table S3.11 Morphometrics of *Xiphinema pachtaicum* (Tulaganov, 1938) Kirjanova, 1951, and *Xiphinema parapachydermum* Gutiérrez-Gutiérrez *et al*. 2012 from several localities in Spain. All measurements in µm and in the format: mean ± SD (range)*.

	X. pachtaicum **(ST80)**	***X. pachtaicum*** **(AR44)**	***X. parapachydermum*** **(AR62)**	***X. parapachydermum*** **(ST122)**
Character/life-stage	**Females**	**Females**	**Females**	**Females**
n	10	10	8	7
L	1640 ± 88 (1488-1788)	1721 ± 79 (1616-1844)	1848 ± 135 (1633-2027)	1854 ± 119 (1695-2017)
a	56.7 ± 3.9 (49.8-61.7)	62.6 ± 3.8 (57.9-67.6)	56.3 ± 2.2 (53.6-60.4)	61.3 ± 1.7 (58.9-63.8)
b	6.8 ± 1.7 (5.0-10.3)	7.5 ± 1.6 (4.3-9.4)	6.6 ± 0.3 (6.2-7.1)	7.3 ± 0.5 (6.8-7.8)
c	58.6 ± 3.7 (53.4-67.2)	58.3 ± 4.7 (49.6-64.0)	62.5 ± 5.3 (55.4-70.7)	68.2 ± 4.9 (62.1-75.1)
c´	1.6 ± 0.1 (1.4-1.7)	1.6 ± 0.1 (1.4-1.7)	1.6 ± 0.1 (1.5-1.8)	1.6 ± 0.1 (1.5-1.7)
V or T	57.2 ± 1.4 (55.0-60.0)	56.7 ± 1.0 (54.5-57.8)	59.1 ± 1.6 (56.0-61.0)	58.5 ± 2.5 (56.0-61.0)
G_1	-	-	-	-
G_2	-	-	-	-
Odontostyle length	83.3 ± 3.0 (79.5-87.0)	83.2 ± 3.8 (77.5-90.5)	82 ± 2.2 (77.5-84.5)	77.6 ± 1.9 (74.0-79.5)
Odontophore length	48.6 ± 2.7 (45-54)	44.8 ± 1.8 (41.5-48.0)	48.6 ± 2.4 (45.5-52.0)	46.1 ± 3.0 (41-49)
Lip region width	8.5 ± 0.3 (8.0-8.5)	8.5 ± 0.2 (8.0-8.5)	9.0 ± 0.5 (8.0-9.5)	9.0 ± 0.6 (8.5-10.0)
Oral aperture-guiding ring	69.3 ± 5.1 (59.0-76.5)	68.4 ± 6.1 (57.5-76.5)	69.5 ± 3.4 (64.0-73.5)	66.7 ± 3.5 (63-70)
Tail length	28.5 ± 1.3 (26.5-30.5)	30.2 ± 1.4 (27.5-32.5)	29.6 ± 1.2 (27.0-30.5)	27.2 ± 0.9 (26.0-28.5)

Character/life-stage	*X. pachtaicum* (ST80) **Females**	*X. pachtaicum* (AR44) **Females**	*X. parapachydermum* (AR62) **Females**	*X. parapachydermum* (ST122) **Females**
Spicules	-	-	-	-
Lateral accessory piece	-	-	-	-

[a] Measurements are in µm and in the form: mean ± standard deviation (range).
[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

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C3

Molecular phylogenetic analysis and comparative morphology resolve two new species of olive-tree soil related dagger nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) from Spain

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Abstract

The genus *Xiphinema* constitutes a large group of ~275 species of polyphagous, plant-ectoparasitic nematodes that are distributed almost worldwide. Some species affect agricultural crops by feeding directly on root cells as well as by transmitting nepoviruses. Despite their agricultural importance, species discrimination in *Xiphinema* is difficult, leading to potential misidentification. Integrative taxonomy, based on the combination of molecular analyses and morphology, constitutes a new insight into *Xiphinema* species identification. In this study we describe two new species of *Xiphinema* from the Iberian Peninsula (***X. macrodora* sp. nov.** and ***X. oleae* sp. nov.**) associated with cultivated and wild olive trees. Both species have specific rRNA sequences. Morphologically, ***Xiphinema macrodora* sp. nov.** is characterised by a very long body (7.2–8.7 mm), a very long odontostyle and odontophore (190–206 and 105–120 mm, respectively), and a well-developed pseudo-Z-organ, comprising 8 to 12 sclerotised bodies. ***Xiphinema oleae* sp. nov.** is characterised by an odontostyle and an odontophore 136–149 and 65–80 mm long, respectively, and a well-developed Z-organ with refractive inclusions (3–5), variable in shape. Additionally, ***X. macrodora* sp. nov.** has the longest body size, and the longest odontostyle and odontophore of any *Xiphinema*, whereas ***X. oleae* sp. nov.** is the first species with a well-developed Z-organ from the Iberian Peninsula.

ADDITIONAL KEYWORDS: Bayesian inference – D2-D3 rRNA – integrative taxonomy ITS.

1. Introduction

The phylum Nematoda comprises some of the most abundant metazoans on earth, with a global distribution and estimated number of species of ~100 000 (Boucher and Lambshead 1995, Blaxter *et al.* 1998, Coomans 2000). In addition, nematodes represent one of the most diverse animal phyla, being ubiquitous in the soil environment (Ferris *et al.* 2001). Probably more than four out of five metazoan individuals on earth are nematodes, and although no recent studies exist in this regard, more than 27 000 species have been described to date (Bongers and Bongers 1998, Hugot *et al.* 2001). In fact, if one compares the number of estimated living species with the number of species that have already been described the Nematoda is the animal group needing the greatest taxonomic effort (Lambshead 1993, Coomans 2000).

The family Longidoridae Thorne, 1935 comprises a wide and diverse array of migratory ectoparasitic nematode species, with dagger nematodes of the cosmopolitan genus *Xiphinema* Cobb, 1913 being one of the most diverse groups (Coomans 1996, Lamberti *et al.* 2000). Damage by *Xiphinema* spp. to host plants is caused by direct feeding on root cells as well as by transmitting nepoviruses (genus *Nepovirus*, family Comoviridae) (Taylor and Brown 1997). In this regard, some species are vectors of several important plant viruses that cause significant damage to a wide range of crops (i.e. *Arabic mosaic virus* (*ArMV*), *Grapevine fanleaf virus* (*GFLV*), *Strawberry latent ringspot virus* (*SLRV*), *Cherry leaf roll virus* (*CLRV*), or *Peach rosette mosaic virus* (*PMV*) (Taylor and Brown 1997). This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* vectors. However, to date only nine of the ~275 known species of *Xiphinema* have been studied and shown to transmit nepoviruses (Decraemer and Robbins 2007).

Due to the large morphological diversity, *Xiphinema* was divided into two species groups (Loof and Luc 1990, Luc *et al.* 1998, Lamberti *et al.* 2000, Coomans *et al.* 2001): (1) the *Xiphinema americanum*-group, which comprises a complex of ~60 species, many of them with a cosmopolitan distribution, characterised by a spiral or C-shaped medium to small body, female reproductive system with two equally developed genital branches usually composed of short uteri without uterine differentiation, and short

conical to broadly convex–conoid tail shape; and (2) the *X.* non-*americanum*-group, which comprises a complex of ~215 species, characterised by a longer body and odontostyle length, different types of development and structures of the two female genital branches, usually constituted with long uteri and uterine differentiation (including the 'Z-organ', spines and/or crystalloid structures in the tubular part of the uterus), and a wide diversity of tail shapes. The high number of species within the *X.* non-*americanum*-group makes species identification difficult in the absence of good diagnostic characters (Loof and Luc 1990, Loof *et al.* 1996). In this respect, no other genera belonging to the order Dorylaimida can compete with *Xiphinema*, specifically the *X.* non-*americanum*-group, in the diversity of the female reproductive system (Coomans *et al.* 2001). It is for this reason, and also for a pragmatic diagnosis, that the group was divided into eight morphospecies groups based on the structural diversity of the female reproductive system and female tail shape (Loof and Luc 1990).

To date, 86 *Xiphinema* species (more than 30% of total nominal species) have been characterised using molecular data, constituting a complementary useful tool to distinguish amongst *Xiphinema* spp. In fact, several recent taxonomic and systematic studies on *Xiphinema* have revealed the existence of complex cryptic species, i.e. species that are morphologically almost identical but genetically distinct (Oliveira *et al.* 2006, Barsi and Luca 2008, Gutiérrez-Gutiérrez *et al.* 2010, 2012, Palomares-Rius *et al.* 2014, Archidona-Yuste *et al.* 2016a). Consequently, application of integrative taxonomy provides a useful approach to species delimitation based on integration of different datasets, e.g. morphology and DNA sequences (Palomares-Rius *et al.* 2014). In fact, integrative taxonomy has been efficiently applied to the rapid and accurate identification of this complex and homogeneous species group (Palomares-Rius *et al.* 2014). Importantly, accurate identification is essential for the selection of appropriate control measures against plant pathogenic or virus-vector species, as well as a reliable method allowing distinction between species under quarantine or regulatory strategies. Ribosomal RNA genes encoding small subunit (SSU) or 18S rRNA, large subunit (LSU) or 28S rRNA, and the internal transcribed spacer 1 (ITS1) region have been used as meaningful genetic markers for the molecular characterisation of species and for resolving phylogenetic relationships within Longidoridae (Ye *et al.*

2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2010, 2011, 2012, Archidona-Yuste *et al.* 2016b). The D2–D3 expansion segments of 28S rRNA and ITS1 rRNA are more useful for species identification than partial 18S rRNA, since the previous two markers show more variability than partial 18S rRNA which, in some cases, does not have sufficient resolution to distinguish species (Neilson *et al.* 2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2011, 2013a). Nevertheless, the partial 18S rRNA gene has also been shown to be useful for discriminating among some *X. americanum*-group species (Lazarova *et al.* 2006).

A survey of nematodes from olive tree soil, including both wild and cultivated trees, conducted in southern Spain, revealed infestations of 1–12 nematodes per 500 cm^3 of soil of four unidentified populations of dagger nematodes belonging to the *X.* non-*americanum*-species group. Preliminary morphological observations indicated that one population appeared morphologically to match morphospecies Group 4 (characterised by equal genital branches in the female and the presence of a Z-organ), while the other three populations were assigned to morphospecies Group 5 (characterised by equal genital branches in the female and the presence of a pseudo-Z-organ or of a pseudo-Z-organ plus uterine spines) (Loof and Luc 1990), being preliminarily identified as the same morphotype. Detailed observations using light microscopy and molecular characterisation indicated that these populations should be assigned to two new species. In the present study we describe these two new species as *Xiphinema macrodora* sp. nov. and *Xiphinema oleae* sp. nov. and present a phylogenetic analysis that confirms that they are unrelated.

The objectives of this study were: (1) to characterize morphologically and morphometrically the two new *Xiphinema* non-*americanum*-species and compare them with previous records; (2) to characterise molecularly the two sampled populations using the D2–D3 expansion segments of the 28S rRNA, ITS1, and partial 18S rRNA gene sequences; and (3) to study the phylogenetic relationships of the *Xiphinema* species with available sequenced species.

2. Material and Methods

2.1 Nematode populations and morphological studies

Nematode surveys were conducted in the spring of 2014 and 2015 in soil of cultivated and wild olive orchards in southern Spain (Table 4.1). Soil samples were collected with a shovel from the upper 50 cm of soil from four or five plants arbitrarily chosen in each locality. Nematodes were extracted from 500 cm3 of soil by centrifugal flotation (Coolen 1979) and a modification of Cobb's decanting and sieving (Flegg 1966) methods. In some cases, additional soil samples were collected afterwards from the same locality for additional specimens for morphological and/or molecular identification.

Specimens for light microscopy were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and embedded in pure glycerine using Seinhorst's (1966) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1000x magnification. Morphometric study of each nematode population included classical diagnostic features of the Longidoridae (i.e. de Man body ratios, lip region and amphid shape, oral aperture-guiding ring, odontostyle and odontophore length: see Jairajpuri and Ahmad (1992). All measurements were expressed in micrometres (mm), unless otherwise indicated in the text. For line drawings of the new species, light micrographs were imported to CorelDraw ver. X5 and redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992).

2.2 DNA extraction, PCR and sequencing

For molecular analyses and in order to avoid mistakes in the case of mixed populations in the same sample, two live nematodes from each sample were temporary mounted in a drop of 1M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed in form to the unidentified populations of *Xiphinema*.

Morphometrics and photomicrographs recorded during this initial study were not used as part of the morphological study or analyses. Following morphological confirmation, the specimens were removed from the slides and DNA extracted. DNA was extracted from single individuals and PCR assays were conducted as described by Castillo *et al.* (2003). One nematode specimen of each sample was transferred to an Eppendorf tube containing 16 mL ddH2O, 2 mL 10 PCR buffer and 2 mL proteinase K (600 mg mL^{-1}) (Promega, Benelux, The Netherlands) and crushed during 2 min with a microhomogeniser, Vibro Mixer (Zürich, Switzerland). The tubes were incubated at 65 ºC (1 h), then at 95 ºC (15 min), and finally at 80 ºC (15 min). Then 1 mL of extracted DNA was transferred to an Eppendorf tube containing: 2.5 mL 10 NH4 reaction buffer, 0.75 mL MgCl2 (50mM), 0.25 mL dNTPs mixture (10 mM each), 0.75 mL of each primer (10 mM), 0.2 mL BIOTAQ DNA Polymerase (BIOLINE, UK) and ddH2O to a final volume of 25 mL. The D2–D3 expansion segments of 28S rRNA was amplified using the D2A (50-ACAAGTACCGTGAGGGAAAGTTG-30) and D3B (50-TCGGAAGGAACCAGCTACTA-30) primers (Nunn 1992). The ITS1 region was amplified using forward primer 18S rRNA (50TTG ATT ACG TCC CTG CCC TTT-30) (Vrain *et al.* 1992) and reverse primer rDNA1 (50-ACG AGC CGA GTG ATC CAC CG-30) (Cherry *et al.* 1997). Finally, the portion of 18S rRNA was amplified using primers 988F (50-CTC AAA GAT TAA GCC ATG C-30), 1912R (50-TTT ACG GTC AGA ACT AGG G-30), 1813F (50-CTG CGT GAG AGG TGA AAT-30) and 2646R (50-GCT ACC TTG TTA CGA CTT TT-30) (Holterman *et al.* 2006).

PCR cycle conditions were: one cycle of 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, annealing temperature of 55 ºC for 45 s, 72 ºC for 3 min, and finally one cycle of 72 ºC for 10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred to above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit V /italic>3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 4.1.

Table 4.1 Taxa sampled for *Xiphinema* species and sequences used in this study –, not obtained or not performed

Species	Sample code	Group[a]	Locality	Host-plant	*D2–D3*	*ITS1*	Partial *18S*
Xiphinema macrodora, sp. nov.	JAO06	5	La Granjuela (Córdoba, Spain)	Cultivated olive	KU171040 KU171041 KU171042	KU171048	KU171052
Xiphinema macrodora, sp. nov.	JAO47	5	Santa Olalla del Cala (Huelva, Spain)	Cultivated olive	KU171043 KU171044	KU171049	–
Xiphinema macrodora, sp. nov.	AR097	5	Santa Mª de Trassierra (Córdoba, Spain)	Wild olive	KU171045	KU171050	–
Xiphinema oleae, sp. nov.	AR035	4	Tarifa (Cádiz, Spain)	Wild olive	KU171037 KU171038 KU171039	KU171046 KU171047	KU171051

[a]Morphospecies group according to Loof and Luc (1990).

2.3 Phylogenetic analysis

D2–D3 expansion segments of 28S rRNA, ITS1, and partial 18S rRNA sequences of different *Xiphinema* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen following previous published studies (He *et al.* 2005, Holterman *et al.* 2006, Gutiérrez-Gutiérrez *et al.* 2013b, Tzortzakakis *et al.* 2015). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT V.7.205 (Katoh and Standley 2013), which accounts for secondary RNA structure. Sequence alignments were visualised using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences + 1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Percentage similarity between sequences was calculated using a sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all gap or place-holding characters were treated as a gap.

When the same position for both sequences had a gap it was not treated as a difference. Phylogenetic analyses of the sequence datasets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fit model of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba *et al.* 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then given to MrBayes for the phylogenetic analyses. Unlinked general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2–D3 expansion segments of 28S rRNA, a transitional model with invariable sites and a gamma-shaped distribution (TIM3 + I + G) for the ITS1 region, and a general time-reversible model with invariable sites and a gamma correction for the partial 18S rRNA were run with four chains for 2 106, 1 106, and 2 106 generations, respectively. A combined analysis of the three genes was not undertaken due to some sequences not being available for all species. The Markov Chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees from all analyses were visualised using TreeView (Page 1996) and FigTree software V.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1 Molecular characterisation of *Xiphinema macrodora* sp. nov. and *Xiphinema oleae* sp. nov.

The amplification of D2–D3 expansion segments of 28S rRNA, ITS1 and the partial 18S rRNA region yielded single fragments of ~800 bp, 1030 bp and 1600 bp, respectively, based on gel electrophoresis. *X. macrodora* sp. nov. and *X. oleae* sp. nov. matched well with the *X.* non-*americanum* group spp. Sequences deposited in GenBank. Nine new D2–D3 of 28S rRNA

gene sequences were obtained in the present study. D2–D3 expansion segments of 28S rRNA sequences of *X. macrodora* sp. nov. (KU171040–KU171045) were related by sequence similarity (95% similarity with substitutions ranged from 28 to 36 and from 8 to 10 indels) with *X. diversicaudatum* (JQ780360–JQ780366), *X. baetica* (KC567166–KC567169) and *X. bakeri* (KF292278). Intraspecific sequence diversity (uncorrected p-distance) of specimens from *X. macrodora* sp. nov. (KU171040–KU171042) studied in the type locality ranged from 0 to 0.10% (1 substitution), and among localities (KU171043–KU171045) ranged from 0.25 to 0.40% (2–3 substitutions). D2–D3 expansion segments of 28S rRNA sequences of *X. oleae* sp. nov. (KU171037–KU171039) were related to *X. turcicum* with a similarity value of 91% (71 substitutions and 6 indels), similarity values with the rest of *X.* non-*americanum*-group spp. ranged from 88% to 86% (from 71 to 106 substitutions from 6 to 23 indels). Intraspecific sequence diversity (uncorrected p-distance) of specimens from *X. oleae* sp. nov. (KU171037–KU171039) studied in the type locality ranged from 0.13% (1 substitution) to 1.1% (8 substitutions). Similar results to D2–D3 were obtained for ITS1 sequences of *X. macrodora* sp. nov. These sequences were related to *X. baetica* (KC567156–KC567157), *X. bakeri* (AF511426) and *X. diversicaudatum* (AJ437027, HG969304), with 87, 86 and 82% similarity, respectively (145, 149 and 209 substitutions and 62, 66 and 79 indels, respectively). Intraspecific variation of ITS1 for *X. macrodora* sp. nov. among the three studied populations (KU171048–KU171050) was low (99% similarity with 2 or 3 nucleotide differences and 0 or 1 indels). *X. oleae* sp. nov. ITS1 showed little sequence similarity with *X.* non-*americanum*-group spp. deposited in GenBank, *X. turcicum* (GU725064) being the closest species, with 83% similarity but only with 54% of coverage. Intraspecific variation of ITS1 detected amongst the two studied specimens from the same locality was also low (99% similarity with 8 nucleotides and no indels).

Finally, the partial 18S rRNA sequences for *X. macrodora* sp. nov. (KU171052) and *X. oleae* sp. nov. (KU171051) showed high similarity (99% p-distance) with several *X. n*on-*americanum*-group spp. deposited in GenBank, including *X. turcicum* (KJ802900), *X. diversicaudatum* (KJ802901), *X. vuittenezi* (EU614267), *X. baetica* (GU725080) and *X. bakeri* (KM199695).

Figura 4.1: Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion segments of 28S rRNA sequence alignment under the general time-reversible model with correction for invariable sites and a gamma-shaped distribution (GTR + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

3.2 Phylogenetic relationships of *Xiphinema macrodora* sp. nov. and *Xiphinema oleae* sp. nov. within the genus *Xiphinema*

Phylogenetic relationships among *X.* non-*americanum*-group species inferred from analyses of D2–D3 expansion segments of 28S rRNA, ITS1 and the partial 18S rRNA gene sequences using BI are given in Figure 4.1-4.3, respectively. Figure 4.1 presents the phylogenetic position for *X. macrodora* sp. nov. and *X. oleae* sp. nov. based on D2–D3 expansion segments of 28S rRNA gene of a multiple-edited alignment (77 sequences) of 756 total characters. The 50% majority rule BI consensus tree of *Xiphinema* spp. showed two well supported major clades (Figure 4.1). Clade (i) was formed by 26 species, including morphospecies from Groups 1, 4, 5, 6, 7 and 8. Group 4 included the only sequenced species *X. oleae* sp. nov. (KU171037–KU171039). The new species from Spain, *X. oleae* sp. nov. (KU171037–KU171039) occupied a basal position in this clade and formed a well-supported subclade (PP = 0.98) with *X. turcicum* (KC567185, GU725077) and *X. lupini* (KC567183, HM921352) but it was clearly separated from both of them. *X. macrodora* sp. nov. (KU171040–KU171045) was placed in the second major clade, Clade (ii). This clade grouped 15 species, 13 of them from Group 5, including the new species described here, *X. macrodora* sp. nov. (KU171040–KU171045), one species from Group 7 and one species from Group 8. *X. macrodora* sp. nov. formed a well-supported subclade (PP = 1.00) with *X. baetica* (KC567167–KC567169). Difficulties were experienced with alignment of the ITS1 sequences due to low similarity. Thus, only related sequences were used in our study (Figure 4.2). The alignment generated for the 28 sequences of ITS1 of *Xiphinema* was 854 bp after discarding ambiguously aligned regions from the alignment. The 50% majority-rule BI consensus tree of *Xiphinema* spp. showed two well supported major clades (Figure 4.2), with PP of 1.00 and 0.98, respectively). Clade (i) was formed only by species from Group 5, including *X. macrodora* sp. nov. (KU171048–KU171050), which formed a well-supported clade (PP = 1.00) with *X. baetica* (KC567156) and it was related to *X. turdetanense* (KC567163). Clade (ii) was formed by six *X.* non-*americanum*-group species from different morphospecies Groups (1, 4, 5, and 7), including *X. oleae* sp. nov. (KU171046–KU171047), which formed a well-supported clade (PP = 1.00) with *X. hunaniense* (EF188844–EF188845) and *X. turcicum*

(GU725064), and a well-supported subclade (PP = 0.99) with *X. chambersi* (AY563427–HM13850), *X. insigne* (AY563427, AY553980) and *X. lupini* (HM921336). These results agree with those obtained for D2–D3 segments (Figure 4.1).

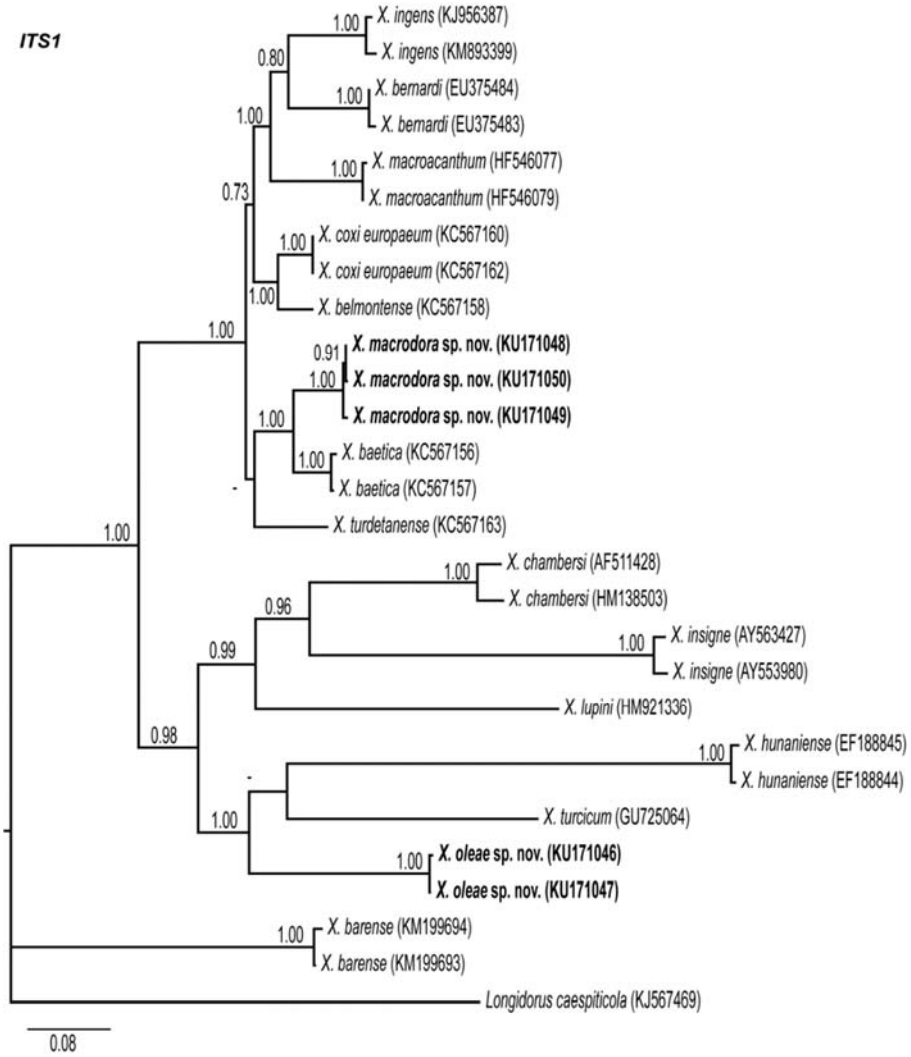

Figura 4.2: Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from ITS1 rRNA sequence alignment under a transitional model of sequence evolution with a correction for invariable sites and a gamma-shaped distribution (TIM3 + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

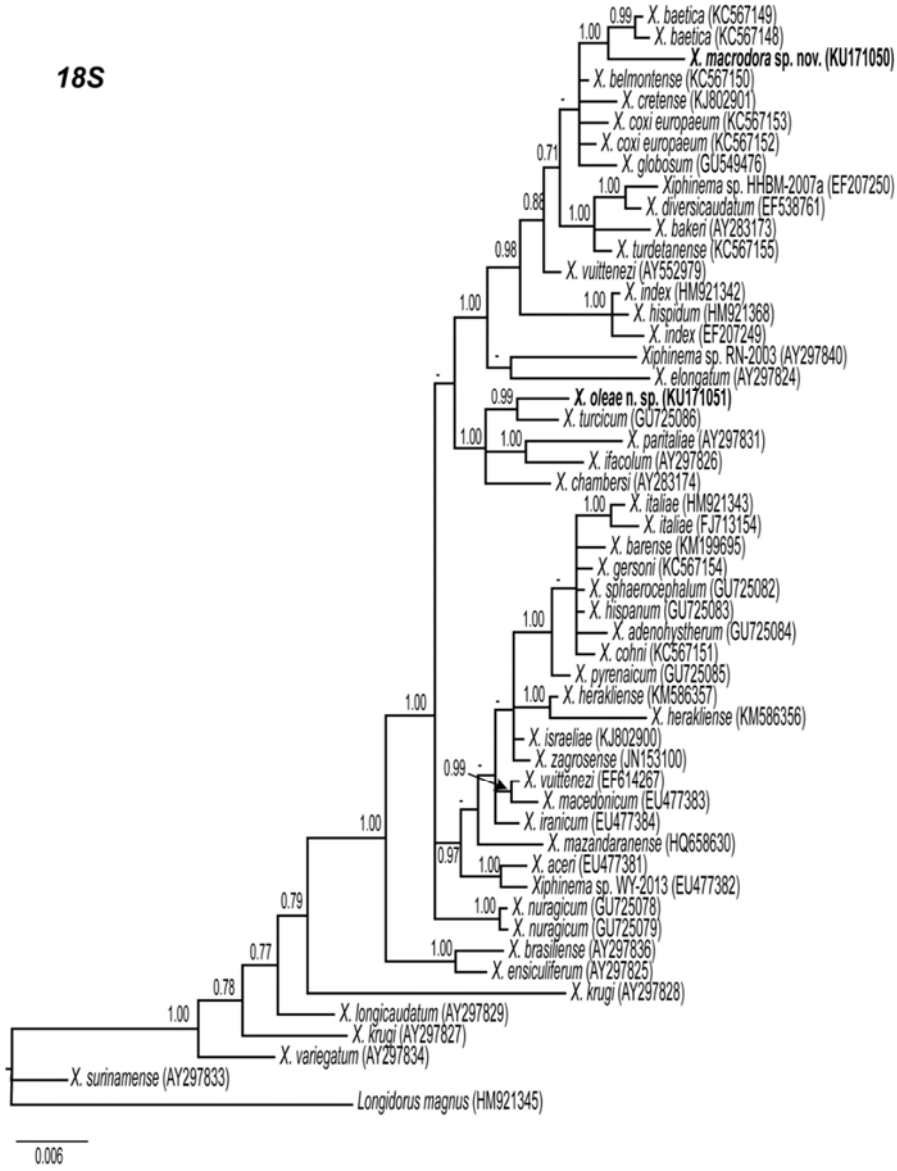

Figura 4.3: Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from 18S rRNA gene sequence alignment under the general time-reversible model of sequence evolution with correction for invariable sites and a gamma-shaped distribution (GTR + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

The 50% majority-rule BI tree of a multiple sequence alignment including 52 partial 18S rRNA sequences of 1545 bp was similar to that obtained in previous studies (Gutiérrez-Gutiérrez *et al*. 2013b, Tzortzakakis *et al*. 2015). For this region, *X. macrodora* sp. nov. (KU171052) and *X. oleae* sp. nov. (KU171051) clustered within the same major clade but in different subclades (Figure 4.3). As in the D2–D3 and ITS1 trees, *X. macrodora* sp. nov. (KU171052) formed a well-supported (PP = 1.00) subclade with *X. baetica* (KC567148–KC567149), and *X. oleae* sp. nov. (KU171051) clustering with *X. turcicum* (GU725086) and *X. ifacolum* (AY297826), another species belonging to Group 4.

3.3 Taxonomy

3.3.1 *Xiphinema macrodora* sp. nov.

http://zoobank.org/urn:lsid:zoobank.org:act:0A94CE3E-98D6-4002-A749-D0A4990235E9

Material examined

Holotype:

Female extracted from soil samples collected from the rhizosphere of cultivated olive trees in La Granjuela, Córdoba province, Spain (38º 22'33.90'N, 5º20'46.90'W), 21.i.2015, by A. Martín Barbarroja and G. León Ropero, mounted in pure glycerine and deposited in the nematode collection at the Institute for Sustainable Agriculture (IAS) of the Spanish National Research Council (CSIC), Córdoba, Spain (collection no. JAO6-20).

Paratypes:

Female, and juvenile paratypes extracted from soil samples collected from the rhizosphere of cultivated olive trees in La Granjuela, Córdoba province, Spain (38º 22' 33.90'N, 5º 20' 46.90'W), and from two additional

populations in Santa Olalla del Cala (Huelva, Spain) and Santa María de Trassierra (Córdoba, Spain), associated with cultivated and wild olive trees, respectively, were deposited in the following nematode collections: IAS-CSIC (collection nos JAO6-01–JAO6-16); one female and one first-juvenile stage at the nematode collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (JAO6-17); one female and one first-juvenile stage at Royal Belgian Institute of Natural Sciences (RBINS), Brussels, Belgium (RIT846); and one female and one first-juvenile stage at the USDA Nematode Collection, Beltsville, MD, USA (T-6687p).

Diagnosis

Xiphinema macrodora sp. nov. is an apparently parthenogenetic species characterised by a very long body (7.2–8.7 mm), assuming a closed C-shaped body when heat relaxed; lip region rounded-hemispherical, separated from body contour by a shallow depression; a very long odontostyle and odontophore, 190–206 and 105–120 mm, respectively; vulva position at 50–55%; well-developed pseudo-Z-organ, comprising 8–12 sclerotised bodies of variable size, and spiniform structures and crystalloid bodies in the uterus; female tail short, dorsally convex–conoid with rounded end, bearing three or four pairs of caudal pores, and shorter than anal body diameter (0.7–0.9); and specific D2–D3, ITS1, and 18S-rDNA sequences with GenBank accession numbers KU171040–KU171042, KU171048–KU171050, and KU171052, respectively. According to the polytomous key by Loof and Luc (1990) and the supplement by Loof *et al*. (1996), the new species belongs to the *X.* non-*americanum* Group 5 and has the following specific a-numeric codes: A4, B2+3, C6, D6, E6, F5, G4, H2, I3, J5, K2, L1.

Description

Female:

Body very long, cylindrical, tapering towards anterior end, and closed C-shaped upon fixation. Cuticle wide, varying to 5.5–7.0 mm at mid-body, and 10.0–12.0 mm at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region rounded-hemispherical, separated from body contour by a shallow depression and 2.4–3.2 times as high as wide. Amphidial fovea stirrup-shaped; aperture extending for 66.7–72.2% of lip region width and located slightly anterior to depression marking lip region. Three pairs of body pores present between anterior end and guiding ring. Odontostyle typical of genus, very long and slender, 10.7 (10.1–11.2) times lip region diameter or 1.7 (1.6–1.9) times odontophore lengths long. Odontophore with well developed flanges 17.4 (14.5–20.0) mm wide. Guiding ring double, guiding sheath 21–28 mm long depending on degree of protraction/retraction of stylet. Pharynx consisting of an anterior slender narrow part, 598 (491–741) mm long, extending to a terminal pharyngeal bulb, 166 (153–174) mm long, with three nuclei. Nucleus of dorsal gland (DN) large, located at 8.4% (6.8–11.4%) of pharyngeal bulb length, being larger than the two ventrosublateral nuclei (S1N) located at 53.5% (50.7–56.9%) of terminal bulb length (location of gland nuclei according to Loof and Coomans (1972). Cardia conoid-rounded, 14.0–16.0 mm long. Intestine simple, prerectum 6.9–10.3 times anal body diameter long, and rectum 0.8–1.0 times anal body diameter long. Female reproductive system didelphic–amphidelphic with branches about equally developed with vulva slit-like, situated posteriorly to mid-body. Each branch composed of an ovary 149–199 mm long, a reflexed oviduct 371–444 mm long with well developed *pars dilatata oviductus* separated from uterus by a well developed sphincter, and a tripartite uterus, 367–488 mm long, composed of *pars dilatata uteri* followed by a tubular part containing in the proximal part a well developed pseudo-Z-organ with weakly muscularised wall, comprising 6–8 sclerotised bodies of variable size, each one consisting of a large central portion, irregularly spherical surrounded by a variable number of refractive pieces, and petal shaped (Figures 4.4D and 4.5E-J). Spiniform structures, variable in length but some of them very large, mixed with small crystalloid bodies, lower in number, distributed over the entire length of the tube-like portion of the uterus (Figure 4.5I). No sperm observed in the

female genital tract. Ovejector well developed (53–86 mm wide), vagina perpendicular to body-axis, extending for 33–49% of corresponding body diameter, vulva a transverse slit. Tail short, always shorter than anal body diameter, dorsally convex–conoid, with rounded end, lacking a blind terminal canal, and bearing three or four caudal pores.

Juveniles:

All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Robbins *et al.* 1996). Juveniles are similar to adults apart from developed reproductive system, shorter body length, tail shape and presence of replacement odontostyle (Figure 4.6). Tail becomes progressively shorter and stouter in each moult (Figure 4.5, Table 4.2). First-juvenile stage was characterised by the replacement odontostyle tip close to base of functional odontostyle and located at level of odontophore. In J2–J4, replacement odontostyle located at some distance from odontophore base. J1 tail dorsally convex–conoid, tail with clavate cuticular extension and ~3 times as long as the anal body diameter (Figure 4.5, Table 4.2). J2 tail dorsally convex-conoid with a cuticular extension. J3 with conical tail with a rounded end subdigitate extension, and J4 tail more rounded and comparable to that of female in shape (Figure 4.5, Table 4.2).

Etymology

The species name refers to the primary distinguishing character of the long odontostyle (from Greek *macros* = long, and *dorus* = stylet).

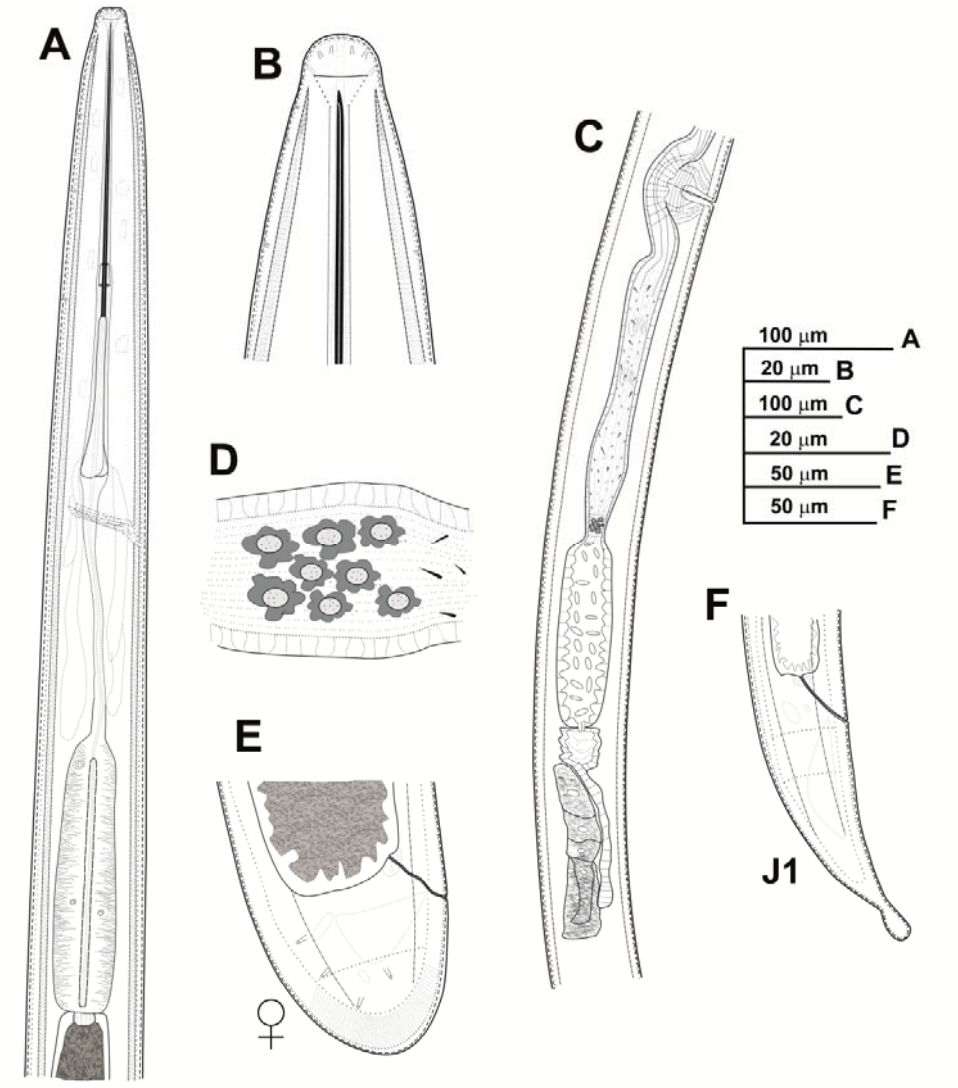

Figura 4.4: Line drawings of *Xiphinema macrodora* sp. nov. A, female neck region. B, female lip region. C, posterior gonad. D, detail of pseudo-Z-organ. E, female tail region. F, first-stage juvenile tail region

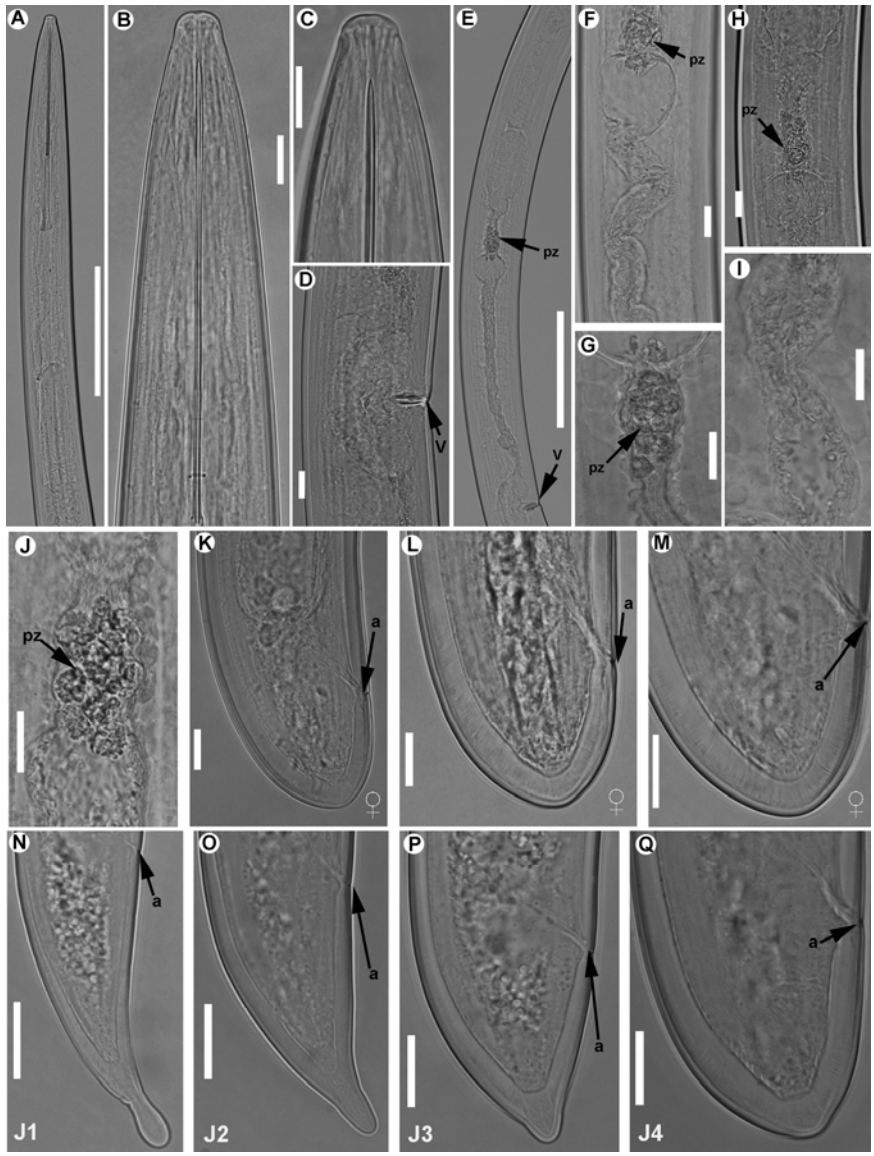

Figura 4.5: Light micrographs of *Xiphinema macrodora* sp. nov. A, female neck region. B, C, female lip region. D, vulval region. E, detail of genital track showing pseudo-Z-organ. F–J, detail of pseudo-Z-organ. K–M, female tail regions. N–Q, first-, second-, third-, and fourth-stage juvenile tails (J1–J4), respectively. Abbreviations: a = anus; pz = pseudo-Z-organ; V= vulva. Scale bars A, E = 200 mm; B–D, G–Q=20 mm.

Relationships

Morphologically, *X. macrodora* sp. nov. belongs to the *X.* non-*americanum* Group 5 of Loof and Luc (1990) and can be differentiated from all known species of the genus by a combination of characters, but particularly by its body and odontostyle length [7.9 (7.2–8.7) mm, 196 (190–206) mm, respectively], the odontostyle being the longest in the genus. Based on the polytomous key by Loof and Luc (1990) and character analysis by Coomans *et al*. (2001), including type of female genital apparatus, uterine differentiation, ratio of tail length to anal body diameter (c'), and body length, it closely resembles *Xiphinema cadavalense* Bravo and Roca 1995, *Xiphinema cretense* Tzortzakakis *et al*. 2014, *Xiphinema coronatum* Roca 1991, and *Xiphinema lusitanicum* Sturhan 1983. *X. macrodora* sp. nov. differs from *X. cadavalense* mainly by having a longer body and odontostyle length [7.9 (7.2–8.7) mm, 196 (190–206) mm, versus 4.8 (4.0–5.3) mm, 158 (151–165) mm, respectively], and female tail shape (dorsally convex–conoid with rounded end versus convex–conoid ending in a peg) (Bravo and Roca 1995). From *X. cretense* it differs mainly in having a longer body and odontostyle length (7.2–8.7 mm, 190–206 mm versus 3.9–6.1 mm, 133–145 mm, respectively), and pseudo-Z-organ with 8–12 granular structures versus 4–6 (Tzortzakakis *et al.* 2014). From *X. coronatum* it differs mainly in having a longer body and odontostyle length (7.2–8.7 mm, 190–206 mm versus 3.8–4.6 mm, 147–157 mm, respectively), and female tail shape (dorsally convex–conoid versus hemispherical tail shape) (Roca 1991). Finally, from *X. lusitanicum* it differs mainly by having a longer body and odontostyle length (7.2–8.7 mm, 190–206 mm versus 4.4–5.9 mm, 168–175 mm, respectively), and female tail shape (dorsally convex–conoid with rounded end versus convex–conoid ending in a peg) (Sturhan 1983).

In addition, *X. macrodora* sp. nov. is molecularly related to *Xiphinema baetica* Gutiérrez-Gutiérrez *et al*. 2013, and *Xiphinema turdetanense* Gutiérrez-Gutiérrez *et al*. 2013, but it can be differentiated on the important diagnostic characters discussed below. From *X. baetica* it differs mainly in uterine differentiation type (pseudo-Z-organ with presence versus absence of spiniform structures), a longer body and odontostyle length (7.2–8.7 mm,

190–206 mm versus 4.9–6.1 mm, 142–157 mm, respectively), female tail shape (dorsally convex–conoid with rounded end versus convex–conoid with distinctly digitate terminus), and absence versus presence of males. On the other hand, it differs mainly from *X. turdetanense* in having a longer body and odontostyle length (7.2–8.7 mm, 190–206 mm versus 4.1–5.2 mm, 121–142 mm, respectively), and female tail shape (dorsally convex–conoid with rounded end versus conoid with digitate or subdigitate terminus) (Gutiérrez-Gutiérrez *et al.* 2013b).

Morphological and morphometric characters of the two additional populations of *X. macrodora* sp. nov. from Santa Olalla del Cala, Huelva province, Spain and Santa María de Trassierra, Córdoba province, Spain, associated with cultivated and wild olive trees, respectively, agree well with those of the type population (Tables 4.2 and 4.3).

3.3.2 *Xiphinema oleae* sp. nov.

http://zoobank.org/urn:lsid:zoobank.org:act:3C62CE81-62C0-4498-9D30-B91D2DC125F3

Material examined

Holotype:

Female extracted from soil samples collected from the rhizosphere of wild olive trees in Tarifa, Cádiz province, Spain (36º 07' 13.40'N, 5º 43' 17.70'W), 13.v.2014, by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine and deposited in the nematode collection at the Institute for Sustainable Agriculture (IAS) of the Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR35-01).

Paratypes:

Female, and juvenile paratypes extracted from soil samples collected from the rhizosphere of wild olive trees in Tarifa, Cádiz province, Spain (36º 07' 13.40'N, 5º 43' 17.70'W) were deposited in the following nematode collections: IAS-CSIC (collection numbers AR35-02-AR35-10); one female

at the nematode collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (AR35-11); two females at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT847); and one female and one first-, second-, third- and fourth-stage juvenile at USDA Nematode Collection, Beltsville, MD, USA (T-6688p).

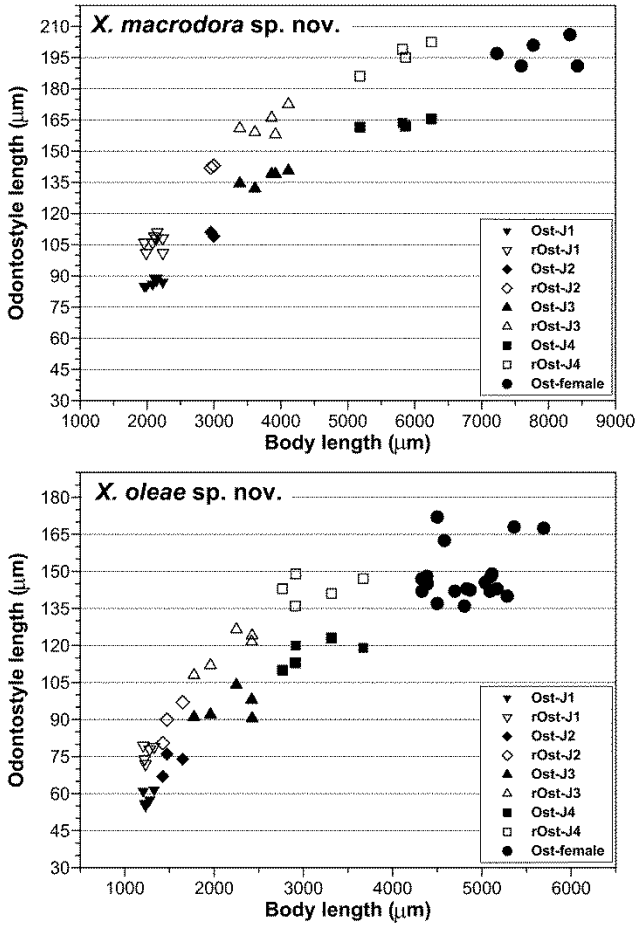

Figura 4.6: Relationship of body length to length of functional and replacement odontostyle (Ost and rOst, respectively); length in all developmental stages from first-stage juveniles (J1) to mature females of: (A) *Xiphinema macrodora* sp. nov. and (B) *Xiphinema oleae* sp. nov.

Table 4.2 Morphometrics of *Xiphinema macrodora*, sp. nov. from cultivated olive at La Granjuela (Córdoba, Spain). Measurements are in micrometres (µm) and in the form: mean ± standard deviation (range)

Characters/ratios	Holotype	Paratypes				
		Females	J1	J2	J3	J4
n	1	22	8	3	5	4
L	8227	7918 ± 453 (7159–8682)	2114 ± 102 (1961–2239)	3060 ± 146 (2954–3227)	3779 ± 283 (3386–4114)	5779 ± 442 (5182–6250)
a (body length/maximum body width)	84.8	77.7 ± 4.9 (68.0–86.0)	48.4 ± 2.2 (45.7–51.3)	58.2 ± 3.5 (54.5–61.5)	63.0 ± 6.1 (57.6–69.6)	64.2 ± 4.8 (61.6–71.4)
b (body length/pharyngeal length)	11.0	11.5 ± 0.7 (10.1–13.1)	6.0 ± 1.0 (4.9–7.7)	7.0 ± 0.8 (6.6–7.9)	6.9 ± 0.1 (6.7–7.0)	9.7 ± 0.8 (8.7–10.8)
c (body length/tail length)	149.6	143.6 ± 8.3 (124.8–155.5)	24.6 ± 1.2 (23.5–25.9)	45.1 ± 2.7 (42.2–47.5)	69.8 ± 4.9 (66.4–75.4)	100.2 ± 3.1 (96.9–104.2)
c′ (tail length/body width at anus)	0.8	0.8 ± 0.04 (0.7–0.9)	2.7 ± 0.1 (2.5–2.8)	1.7 ± 0.1 (1.6–1.8)	1.2 ± 0.1 (1.1–1.3)	1.0 ± 0.03 (0.9–1.0)
V ((distance from anterior end to vulva/body length) × 100)	54.0	53.1 ± 1.2 (50.0–55.0)	–	–	–	–
G_1	8.0	10.9 ± 1.1 (9.6–12.2)	–	–	–	–
G_2	7.4	10.7 ± 1.3 (9.2–12.4)	–	–	–	–
Odontostyle length	192.0	196.3 ± 4.4 (190.0–206.0)	86.9 ± 1.6 (85.0–89.0)	110.0 ± 1.4 (109.0–111.0)	137.0 ± 3.6 (132.0–140.5)	163.1 ± 1.8 (161.5–165.5)
Replacement odontostyle length	–	–	106.3 ± 3.6 (101.0–111.0)	144.3 ± 3.2 (142.0–148.0)	163.3 ± 6.0 (158.0–172.5)	195.6 ± 7.1 (186.0–202.5)
Odontophore length	113.0	115.0 ± 4.5 (105.0–120.0)	55.2 ± 3.4 (51.0–62.0)	78.7 ± 0.6 (78.0–79.0)	86.3 ± 2.9 (83.0–90.5)	102.0 ± 4.4 (95.5–105.0)
Lip region width	18.0	18.4 ± 0.7 (17.0–19.0)	11.3 ± 0.6 (10.5–12.0)	13.0 ± 0.5 (12.5–13.5)	14.8 ± 0.9 (14.0–16.0)	16.6 ± 0.6 (16.0–17.5)
Oral aperture-guiding ring	173.0	185.0 ± 10.7 (165.0–204.0)	74.4 ± 2.5 (70.0–78.0)	105.2 ± 2.8 (102.5–108.0)	132.9 ± 5.8 (125.5–138.0)	158.8 ± 9.6 (146.0–169.0)
Tail length	55.0	55.0 ± 3.3 (47.0–61.0)	83.8 ± 7.3 (72.0–95.0)	67.8 ± 2.3 (65.5–70.0)	55.1 ± 4.4 (52.0–62.0)	57.6 ± 3.7 (52.5–60.5)
J (hyaline tail region length)	12.0	10.8 ± 0.8 (10.0–12.0)	13.6 ± 1.4 (12.0–16.0)	18.5 ± 0.5 (18.0–19.0)	12.1 ± 1.3 (10.5–14.0)	11.3 ± 1.3 (10.0–12.5)

Table 4.3 Morphometrics of *Xiphinema macrodora*, sp. nov. from cultivated olive at Santa Olalla del Cala (Huelva, Spain) and wild olive at Santa Mª de Trassierra (Córdoba, Spain). Measurements are in micrometres (µm) and in the form: mean ± standard deviation (range)

Characters/ratios [b]	Santa Olalla del Cala	Santa Mª de Trassierra
	Females	Females
n	4	3
L	8051 ± 544 (7295–8591)	7745 ± 639 (7068–8341)
a (body length/maximum body width)	72.1 ± 5.1 (64.6–75.8)	77.6 ± 5.2 (72.1–82.6)
b (body length/pharyngeal length)	10.4 ± 1.7 (8.5–12.0)	10.7 ± 1.9 (8.6–12.2)
c (body length/tail length)	128.2 ± 4.9 (123.0–132.6)	144.9 ± 5.8 (141.4–151.7)
c′ (tail length/body width at anus)	0.8 ± 0.1 (0.7–0.9)	0.80 ± 0.05 (0.76–0.85)
V ((distance from anterior end to vulva/body length) × 100)	50.5 ± 1.9 (49.0–53.0)	50.0 ± 1.0 (49.0–51.0)
G_1	11.2 ± 1.2 (9.8–12.0)	10.6 ± 0.9 (9.9–11.2)
G_2	10.9 ± 1.1 (9.8–12.0)	10.3 ± 0.6 (9.9–10.8)
Odontostyle length	201.5 ± 4.8 (195.0–206.0)	197.0 ± 6.6 (190.0–203.0)
Odontophore length	119.8 ± 4.0 (114.0–123.0)	111.5 ± 10.0 (100.0–118.0)
Lip region width	18.3 ± 0.5 (18.0–19.0)	18.7 ± 0.6 (18.0–19.0)
Oral aperture-guiding ring	182.5 ± 9.7 (173.0–186.0)	182.7 ± 11.9 (173.0–196.0)
Tail length	62.9 ± 5.3 (55.0–66.5)	53.3 ± 2.9 (50.0–55.0)
J (hyaline tail region length)	13.5 ± 2.1 (12.0–15.0)	13.7 ± 1.5 (12.0–15.0)

Diagnosis

Xiphinema oleae sp. nov. is an apparently parthenogenetic species characterised by a lip region rounded-hemispherical, separated from body contour by a shallow depression; odontostyle and odontophore 136–149 and 65–80 mm, respectively; vulva position at 50–53%; well developed Zorgan, with heavy muscularised wall, and moderately refractive inclusions, variable in number (3–5) and shape (from round to star-shaped), with clear central area; female tail short, dorsally convex–conoid, with rounded end. Specific D2–D3, ITS1, and 18S rRNA sequences were deposited in GenBank with accession numbers KU171037–KU171039, KU171046–

KU171047, and KU171051, respectively. According to the polytomous key by Loof and Luc (1990) and the supplement by Loof *et al*. (1996), the new species belongs to the *X.* non-*americanum* Group 4 and has the following specific a-numeric codes: A4, B1, C6, D6, E6, F5, G3, H2, I4, J6, K4, L1.

Description

Female:

Body cylindrical, tapering towards anterior end, and open spiral-shaped upon fixation. Cuticle 2.0–3.0 mm at mid-body, and 8.0–13.0 mm at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region rounded, separated from body contour by a shallow depression and 1.6–2.6 times as high as wide. Amphidial fovea stirrup-shaped; aperture extending for 68.1–88.9% of lip region width and located slightly anterior to depression marking lip region. Two pairs of body pores present between anterior end and guiding ring. Odontostyle typical of genus, long and slender, 11.4 (10.5–12.1) times lip region diameter or 2.0 (1.7–2.3) times odontophore length long. Odontophore with well developed flanges 10.9 (9.0–14.5) mm wide. Guiding ring double, and guiding sheath 18–24 mm long depending on degree of protraction/retraction of stylet. Pharynx consisting of an anterior slender narrow part, 401 (298–652) mm long, extending to a terminal pharyngeal bulb, 133 (115–150) mm long, with three nuclei. Nucleus of dorsal gland (DN) large, located at 11.4% (8.2–14.1%) of pharyngeal bulb length, being larger than the two ventrosublateral nuclei (S1N) located at 58.2% (53.0–63.1%) of terminal bulb length (location of gland nuclei according to Loof and Coomans (1972)). Cardia conoid–rounded, 5.0–7.0 mm long. Intestine simple, prerectum of variable length, 11.2–22.4 times anal body diameter long, and rectum 0.6–0.9 times anal body diameter long. Female reproductive system didelphic–amphidelphic with branches about equally developed, with vulva slit-like, situated posteriorly to mid body. Each branch composed of an ovary 79–100 mm long, a reflexed oviduct 102–145 mm long with well developed *pars dilatata oviductus*, and a tripartite uterus 167–198 mm long composed of *pars dilatata uteri* followed by a tubular part containing in the proximal part a well developed Z-organ with heavily muscularised wall, and

moderately refractive inclusions, variable in number (3–5) and shape (from round to star-shaped), with clear central area (Figures 4.7D-F and 4.8F-K). No sperm observed in the female genital tract. Ovejector well developed, 34–56 mm wide, vagina perpendicular to body-axis, extending for 34–51% of corresponding body diameter, and vulva as a transverse slit. Tail short, almost as long as anal body diameter, dorsally convex–conoid, with rounded end, lacking a blind terminal canal, and bearing two or three caudal pores.

Juveniles:

All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Robbins *et al*. 1996). Juveniles similar to adults apart from developed reproductive system, shorter body length, tail shape and presence of replacement odontostyle. Tail becomes progressively shorter and stouter in each moult (Figure 4.7, Table 4.4). First-juvenile stage was characterised by the replacement odontostyle tip close to base of functional odontostyle and located at level of odontophore (Figure 4.8L). In J2–J4, replacement odontostyle located at some distance from odontophore base. J1 tail conoid and ~3 times as long as the anal body diameter (Figure 4.7, Table 4.4). J2 and J3 tail broadly conoid, and J4 tail more rounded and comparable to that of female in shape (Figure 4.7, Table 4.4).

Etymology

The species name is derived from the Latin word *oleae* (genitive feminine) = olive (*Olea europaea* subsp. *sylvestris*), the plant from which the new species was isolated.

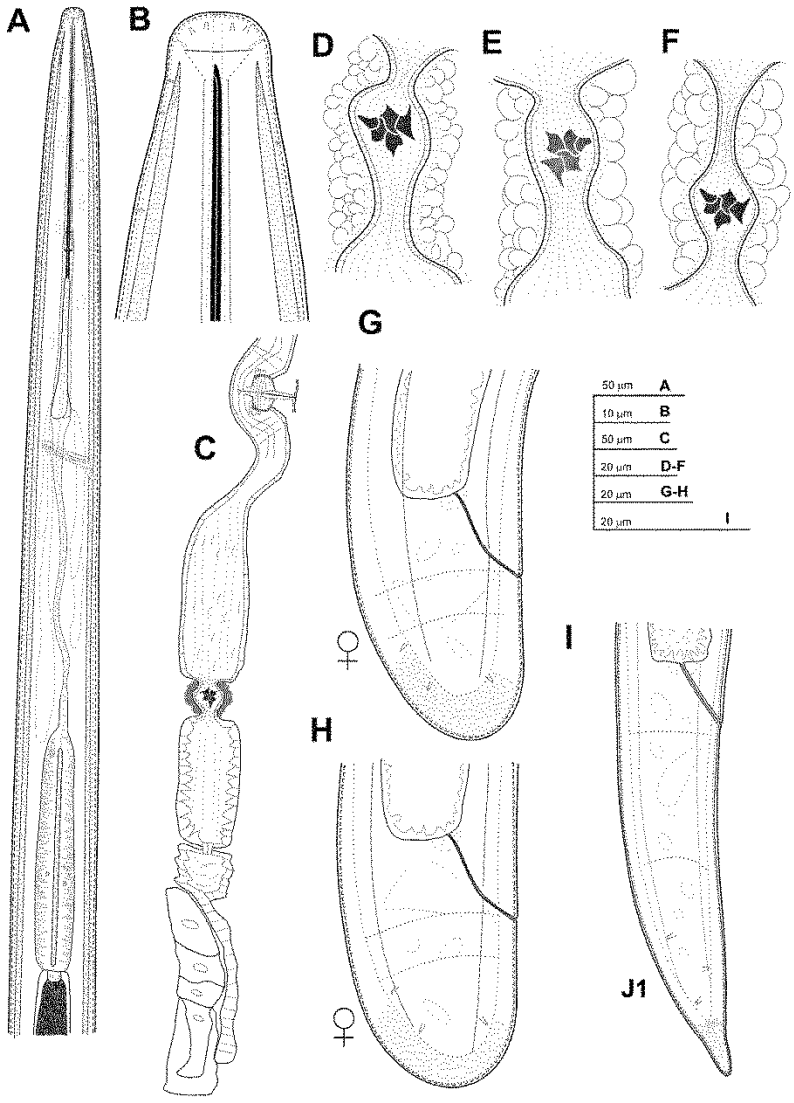

Figura 4.7: Line drawings of *Xiphinema oleae* sp. nov. A, female neck region. B, female lip region. C, detail of genital track showing Z-organ. D–F, detail of Z-organ. G, H, female tail regions. I, first-stage juvenile tail region.

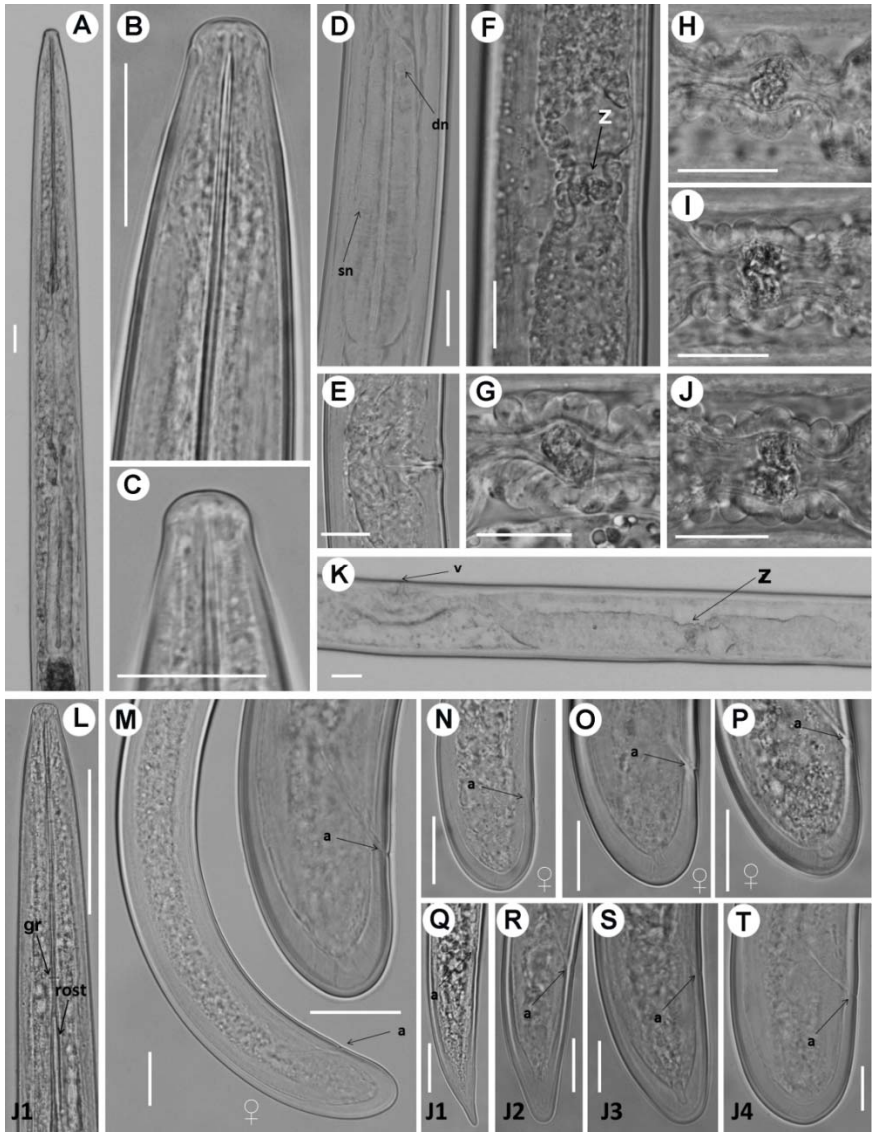

Figura 4.8: Light micrographs of *Xiphinema oleae* sp. nov. A, female neck region. B, C, female lip region. D, pharyngeal bulb. E, vulval region. F–J, detail of Z-organ. K, Detail of genital track showing Z-organ. L, first-stage juvenile neck region. M–P, female tail regions. J, male tail. Q–T, first-, second-, third-, and fourth-stage juvenile tails (J1–J4), respectively. Abbreviations: a = anus; dn = dorsal nucleus; gr = guiding ring; rost = replacement dontostyle; sn = ventrosublateral nucleus; v = vulva; z = Z-organ. Scale bars: A–P=25 mm; Q–T=50 mm.

Relationships

Morphologically, *X. oleae* sp. nov. with a well developed Z-organ belongs to the *X.* non-*americanum* Group 4 in Loof and Luc (1990). Within species of *X.* non-*americanum* Group 4 with rounded non-digitate tail, *X. oleae* sp. nov. closely resembles *Xiphinema algeriense* Luc and Kostadinov 1982, *Xiphinema rotundatum* Schuurmans, Stekhoven and Teunisen 1938, and *Xiphinema tropicale* Zullini 1973. First, *X. oleae* sp. nov. differs from *X. algeriense* mainly in having a longer odontostyle (136–149 mm versus 106–125 mm), posterior vulva position (50–53% versus 47–50%), lower c' ratio (0.8–1.0 versus 1.5–2.1), female tail shape (dorsally convex–conoid with rounded end versus conical with rounded end), and absence versus presence of males (Luc and Kostadinov 1982). From *X. rotundatum* it differs mainly by having a longer body and odontostyle length (4.3–5.3 mm, 136–149 mm versus 2.9–3.4 mm, 122–128 mm, respectively), posterior vulva position (50–53% versus 45–50%), and higher c' ratio (121.6–166.8 versus 55–99) (Schuurmans-Stekhoven and Teunissen 1938). Finally, it differs mainly from *X. tropicale* by having a longer body and odontostyle length (4.3–5.3 mm, 136–149 mm versus 2.2–2.6 mm, 110–122 mm, respectively), posterior vulva position (50–53% versus 37–39%), and higher c' ratio (121.6–166.8 versus 70–112) (Zullini 1973).

4. Discussion

The primary objective of this study was to identify and to characterise morphometrically and molecularly two species of dagger nematodes belonging to the *Xiphinema* non-*americanum* group detected in cultivated and wild olive orchards in Spain, and to assign molecular markers useful for distinguishing among *Xiphinema* species, which may have critical Phytopathological implications. We describe here two new species of *Xiphinema*, belonging to the morphospecies Group 4 and 5 of Loof and Luc (1990), based on integrative taxonomy and their phylogenetic relationships based on nuclear rRNA.

Delimiting closely related *X.* non-*americanum* group species is a particularly difficult issue. The current study has demonstrated the need for an integrative approach by combining molecular techniques with morphology and morphometric measurements. This was very useful for the accurate identification of *Xiphinema* because of the low interpopulation variability found for some species in the D2–D3 and ITS1 rRNA genes (i.e. *X. baetica*, *X. index*) (Gutiérrez-Gutiérrez *et al.* 2010, 2011). The present results expand the known diversity of *X.* non-*americanum* group in the Iberian Peninsula where several species of this group and species from the *X. americanum*-group have been recently described in previous studies (Gutiérrez-Gutiérrez *et al.* 2012, 2013b, Archidona-Yuste *et al.* 2016b). A remarkable result of the present study is the report of a new species of the *X.* non-*americanum* group showing the largest body and stylet length within *Xiphinema* (*X. macrodora* sp. nov.) and another one characterised by the presence of a well developed Z-organ (*X. oleae* sp. nov.), included in morphospecies Group 4, which comprised a lower number of species than in other morphospecies groups (Loof and Luc 1990). The relationship between stylet and nematode body length in several genera of plant-parasitic nematodes is generally positive and significant (Yeates 1986). Functional and developmental aspects of morphometrics of plant-parasitic nematodes have been examined previously and the patterns they demonstrate indicate that relationships among nematode dimensions are deterministic (i.e. non-random) and non-parametric (i.e. not independent) (Geraert 1968, Yeates 1986). In fact, we have detected in the same soil sample (La Grajuela, Córdoba province, Spain) a nematode species of the genus *Longidorus* with a long stylet, which has been recently described as a new species (*Longidorus macrodorus*: Archidona-Yuste *et al*. 2016b). This suggests an adaptive change and a selective advantage by these nematodes in order to exploit different food resources such as a wide host-range of woody roots, as well as inhabiting both natural and agricultural systems, as it is the case of wild and cultivated olives. Additionally, long body size in nematodes could allow for movement deeper

into the soil to avoid dry conditions in summer. How these nematode species (*L. macrodorus* and *X. macrodora* sp. nov.) with long stylets and large body sizes occupy this habitat is difficult to explain, one possibility being that the species were selected in this area by soil type or by host-plant constraints. In marine nematodes, mean nematode length increases

as their depth in the sediment increases (Soetaert *et al.* 2002). *X. macrodora* sp. nov. has been found in two additional localities. Thus, convergent morphological adaptations also appear to have occurred independently in different lineages of plant-parasitic nematodes. However, this point is based on only a few samples found in wild and cultivated olive orchards. The morphospecies Group 4 comprises 16 nominal species, mostly distributed in tropical and subtropical regions of Africa (including Nigeria with four species, Ivory Coast with three species, Guinea, Kenya and northern South Africa, with one species each), tropical regions of the Caribbean (including Saint Lucia in the Lesser Antilles, Guyana, and Mexico, with one species each), and one species in Saudi Arabia (Coomans *et al*. 2001). Two species have been reported from regions with a Mediterranean climate, including Algeria and south-western Bulgaria (Coomans *et al*. 2001). These distributional data suggest that morphospecies Group 4 is associated with warm-humid climate conditions, as reported for other genera such as *Trichodorus* (Decraemer *et al.* 2013) and *Rotylenchus (Cantalapiedra-Navarrete et al. 2013)*.

Sequences of nuclear rRNA genes, particularly D2–D3 and ITS1, have proven to be a powerful tool for providing accurate species identification of Longidoridae (He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2012, Palomares-Rius *et al.* 2013). However, the D2–D3 expansion region was more useful for phylogenetic relationships among *Xiphinema* species than ITS1 because the latter showed high molecular variability. Thus, ITS1 appears better suited for differentiating species than for phylogenetic analysis of *Xiphinema*. Our findings also confirm that partial 18S rRNA sequences do not have enough phylogenetic resolution, because of low variability amongst species. Phylogenetic inferences based on D2–D3, ITS1 and18S rRNA suggest that *X. oleae* sp. nov. and *X. turcicum* are closely related phylogenetically as well as *X. macrodora* sp. nov. and *X. baetica* (described from the Iberian Peninsula). However, these species showed several morphological differences that made it difficult to establish a correspondence between morphological characters and the phylogenetic trees generated from the molecular data, as for example, the presence of a Z-organ or pseudo-Z-organ between X*. oleae* sp. nov. versus *X. turcicum* and from *X. macrodora* sp. nov. versus *X. baetica* in body and odontostyle lengths among other morphometric–morphological characters.

The present data did not show a strong correlation between morphospecies and their grouping in the phylogenetic analysis using molecular markers, a finding already reported by Gutiérrez-Gutiérrez *et al*. (2013b), Roshan-Bakhsh *et al*. (2014) (Roshan-Bakhsh *et al.* 2014) and De Luca *et al*. (2014). Our results on phylogenetic relationships in the *X.* non-*americanum* group inferred from the D2–D3 region of 28S rRNA and partial 18S rRNA trees suggest that the large morphospecies Groups 1, 5, 6 and 7 may be considered paraphyletic, which agrees with the cladistic analysis of Coomans *et al*. (2001). Phylogenetic analyses based on D2–D3, ITS1 and partial 18S rRNA using BI (Figures 4.1-4.3) resulted in a congruent position of the newly sequenced species of the *X.* non-*americanum* group from Spain, which grouped in separate clades, with most species belonging to Groups 4 and 5. In any case, the position of some species is difficult to assign based on analysis of the sequence data deposited in GenBank. The case for *X. diversicaudatum* in D2–D3and ITS1 trees (Figures 4.1 and 4.2), and *X. vuittenezi* in the 18S rRNA tree (Figure 4.3) are paradigmatic examples. A D2–D3 sequence from Slovakia (EF538755) and another from Portugal (AY601624), and an ITS1 sequence from France (AJ437027) and another of unknown origin (AY439183), appeared in different positions in the phylogenetic trees obtained in this study (Figures 4.1 and 4.2). Similarly, *X. pyrenaicum* from Cyprus (AY601627) clustered separately from *X. pyrenaicum* (GU725073, France), but the former species was identified on the basis of 'general morphology' (He *et al.* 2005) and the latter in an integrative study using morphological and molecular data (Gutiérrez-Gutiérrez *et al.* 2010); as well as *X. elongatum* (EF140790, China) and *X. elongatum* (AY601618, Israel) (Figure 4.2); or X. vuittenezi (AY552979) from Kenya and from the Czech Republic (EF614267) (Figure 4.3). These occurrences are good examples demonstrating the difficulties of species identification in this complex genus due to character overlap (He *et al.* 2005), and may also suggest the presence of cryptic species within these species groups (Gutiérrez-Gutiérrez *et al.* 2010). In fact, these data suggest that a population of *X. diversicaudatum* (AY601624) from Portugal, identified on the basis of 'general morphology' (He *et al.* 2005), most probably is a misidentification and should be considered conspecific with *X. coxi europaeum*, since the D2–D3 sequences from the six Spanish populations closely matched (99% similarity) those from the *X. diversicaudatum* population (AY601624, Portugal). These examples demand special attention in assigning molecular markers for *Xiphinema*

spp. Given that some species are virus vectors and, thus, may have critical phytopathological implications. In this respect, *X. diversicaudatum* and *X. coxi coxi* have been reported as vectors of *ArMV*, *SLRV* and *CLRV* (Taylor and Brown 1997).

5. Conclusions

In summary, the present study expands the body size and odontostyle length ranges known for *Xiphinema* spp., as well as the geographical distribution of mophospecies Group 4, showing the plasticity of these nematodes and the importance of describing new species integrating morphometric and molecular approaches. Additionally, *X. macrodora* sp. nov. co-occurred with another large longidorid nematode with a long stylet (*L. macrodorus*) in one sample studied. Further research will be required in order to determine how the presence of these characters relates to host and/or soil ecological traits. The description of these two new species also highlights the presence of nematodes with the pseudo-Z-organ and a well developed Z-organ in the Iberian Peninsula. The function of this organ is still a matter of debate, but it could be related to egg development.

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C4

Remarkable diversity and prevalence of dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches

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Abstract

The genus *Xiphinema* includes a remarkable group of invertebrates of the phylum Nematoda comprising ectoparasitic animals of many wild and cultivated plants. Damage is caused by direct feeding on root cells and by vectoring nepoviruses that cause diseases on several crops. Precise identification of *Xiphinema* species is critical for launching appropriate control measures. We make available the first detailed information on the diversity and distribution of *Xiphinema* species infesting wild and cultivated olive in a wide-region in southern Spain that included 211 locations from which 453 sampling sites were analyzed. The present study identified thirty-two *Xiphinema* spp. in the rhizosphere of olive trees, ten species belonging to *Xiphinema americanum*-group, whereas twenty-two were attributed to *Xiphinema* non-*americanum*-group. These results increase our current knowledge on the biodiversity of *Xiphinema* species identified in olives and include the description of four new species (***Xiphinema andalusiense* sp. nov.**, ***Xiphinema celtiense* sp. nov.**, ***Xiphinema iznajarense* sp. nov.**, and ***Xiphinema mengibarense* sp. nov.**), and two new records for cultivate olives (*X. cadavalense* and *X. conurum*). We also found evidence of remarkable prevalence of Xiphinema spp. in olive trees, viz. 85.0% (385 out of 453 sampling sites), and they were widely distributed in both wild and cultivated olives, with 26 and 17 *Xiphinema* spp., respectively. Diversity indexes (Richness, Hill's diversity, Hill's reciprocal of D and Hill's evenness) were significantly affected by olive type. We also developed a comparative morphological and morphometrical study together with molecular data from three nuclear ribosomal RNA genes (D2-D3 expansion segments of 28S, ITS1, and partial 18S). Molecular characterization and phylogenetic analyses allowed the delimitation and discrimination of four new species of the genus described herein and three known species. Phylogenetic analyses of *Xiphinema* spp. resulted in a general consensus of these species groups. This study is the most complete phylogenetic analysis for *Xiphinema* non-*americanum*-group species to date.

1. Introduction

Soil is most likely one of the more species-rich habitats of terrestrial ecosystems because over one quarter of all living species on Earth are inhabiting the soil (Wolters 2001, Decaëns *et al.* 2006). One of the most diverse soil animals are nematodes although they are ubiquitous in all habitats that provide available organic carbon sources (Bongers and Ferris 1999). The phylum Nematoda includes species either free-living or parasites of animals or plants. Plant-parasitic nematodes (PPN) comprising about 15% of the total number of nematode species currently known, of which over 4,100 species have been identified as PPN (Wyss 1997, Decraemer and Hunt 2006). The fact that new species of PPN are continually being described, combined with PPN gross morphology tends to be highly conserved, likewise the limitations of species concepts, results in an increase of the difficulty in the species identification (Coomans 2000, Siddiqi 2000, Oliveira *et al.* 2006, Subbotin and Moens 2006, Gutiérrez-Gutiérrez *et al.* 2010, Cantalapiedra-Navarrete *et al.* 2013, Palomares-Rius *et al.* 2014, Archidona-Yuste *et al.* 2016c). However, accurate identification of PPN is essential for the selection of appropriate control measures against plant pathogenic species, as well as for a reliable method allowing distinction between species under quarantine or regulatory strategies and a better understanding of their implications in pest control and soil ecology (Coomans *et al.* 2001). Integrative taxonomy has been efficiently applied for the accurate diagnostic and identification over the wide range of PPN species (Ye *et al.* 2004, Gutiérrez-Gutiérrez *et al.* 2010, 2012, 2013b, Cantalapiedra-Navarrete *et al.* 2013, Palomares-Rius *et al.* 2014, Archidona-Yuste *et al.* 2016c, b).

The most important nematodes economically include endoparasitic species such as the root-knot (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.), likewise the ectoparasitic nematodes belonging to the family Longidoridae Thorne, 1935 (Thorne 1935). Dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Cobb 1913) are one of the highest diversified group species of this family (Coomans 1996). The phytopathological importance of this group of nematodes not only lies in its wide range of host and cosmopolitan distribution but some species of this genus are vectors of several important

plant viruses (genus *Nepovirus*, family Comoviridae) that cause significant damage to a wide range of crops (Coomans 1996, Taylor and Brown 1997, Lamberti *et al.* 2000, Macfarlane *et al.* 2002, Macfarlane 2003, Decraemer and Robbins 2007). Considering the great morphological diversity, the genus *Xiphinema* was divided into two different species groups (Loof and Luc 1990, Lamberti *et al.* 2000, Coomans *et al.* 2001): i) the *Xiphinema americanum*-group comprising a complex of about 60 species (Lamberti *et al.* 2000, Archidona-Yuste *et al.* 2016a); and ii) the *Xiphinema* non-*americanum*-group which comprises a complex of more than 215 species (Coomans *et al.* 2001, Gutiérrez-Gutiérrez *et al.* 2013b, Archidona-Yuste *et al.* 2016b). Species discrimination in *Xiphinema* is based mainly on classical diagnostic features; however, due to a high degree of intraspecific morphometric variability can lead to overlapping among *Xiphinema* species and increase the risk of species miss-identification (Loof and Luc 1990, Lamberti *et al.* 2004, Archidona-Yuste *et al.* 2016a).

Recently, 96 *Xiphinema* species (about 35% of total species) have been characterized molecularly by ribosomal genes (D2-D3 expansion segments of 28S rRNA and ITS1 rRNA and partial 18S), constituting a useful tool for molecular-based species identification (Ye *et al.* 2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2010, 2011b, 2012, 2013b, Groza *et al.* 2013, Tzortzakakis *et al.* 2014, Archidona-Yuste *et al.* 2016a, c). *Xiphinema* species identification becomes difficult when dealing with morphological closely species that co-occur in a sample or region, as often detected in the Iberian Peninsula (Gutiérrez-Gutiérrez *et al.* 2013b, Archidona-Yuste *et al.* 2016a). Several authors have highlighted the great diversity of *Xiphinema* spp. detected in the Iberian Peninsula (Peña-Santiago *et al.* 2006, Ali *et al.* 2014, Archidona-Yuste *et al.* 2016a, b, c). In particular around 40 species of the genus *Xiphinema* have been reported in Spain, mainly associated with woody, ornamental and vegetable plant species (Peña-Santiago *et al.* 2006, Gutiérrez-Gutiérrez *et al.* 2010, 2012, 2013a, 2013b, Archidona-Yuste *et al.* 2016b, c).

Olive, in wild and cultivated forms, is widely distributed in the Mediterranean Basin, and particularly in southern Spain (Belaj *et al.* 2007, FAOSTAT 2014, MAGRAMA 2014, Archidona-Yuste *et al.* 2016c).Wild and cultivated olives are hosts and damaged by PPN, including dagger

nematodes (*Xiphinema* spp.) (Castillo *et al.* 2010, Ali *et al.* 2014). However, little information is available about *Xiphinema* spp. associated with olive trees, except for the recent contributions of Archidona-Yuste *et al*. (2016a, b, c) reporting new species such as *Xiphinema macrodora* Archidona-Yuste *et al*. 2016, *Xiphinema oleae* Archidona-Yuste *et al*. 2016, *Xiphinema plesiopachtaicum* Archidona-Yuste et al. 2016, and *Xiphinema vallense* Archidona-Yuste *et al*. 2016. Therefore, with the aim of deciphering the biodiversity of *Xiphinema* spp. infecting wild and cultivated olives in southern Spain, we surveyed a total of 211 localities at the eight provinces of Andalusia where both olive forms were present. This survey raised 385 populations of *Xiphinema* species, apparently morphologically related to other known *Xiphinema* spp. This prompted us to carry out an integrative taxonomic study to identify the species within this complex genus.

The general objectives of this research was to study the occurrence and abundance of *Xiphinema* species and to test the resemblance between morphological and molecular data within *Xiphinema* species, and the specific objectives were: i) to identify the 385 Spanish populations of *Xiphinema* spp. detected in wild and cultivate olives; ii) to carry out a molecular characterization of these *Xiphinema* populations based on sequences of the D2-D3 expansion segments of the 28S nuclear ribosomal RNA gene, the ITS1 of rRNA, and partial 18S rRNA sequences; and iii) to study the phylogenetic relationships of *Xiphinema* spp.

2. Material and Methods

2.1 Ethics Statement

No specific permits were required for the described fieldwork studies. Permission for sampling the olive orchards was granted by the landowner. The samples from wild olives were obtained in public areas, forests, and other natural areas studied and do not involve any species endangered or protected in Spain. The sites are not protected in any way.

2.2 Soil collection and nematode extraction

Nematodes were surveyed from 2012 to 2015 during the spring season in wild and cultivate olives growing in Andalusia, southern Spain (Table 5.1, Figure 5.1). Soil samples were collected for nematode analysis with a shovel from four to five trees randomly selected in each sampling site. A total of 115 and 338 sampling sites from wild and cultivated olives, respectively, were arbitrarily chosen in the eight provinces of Andalusia where both olive subspecies were present. The number of sampling sites was proportional to the area of wild and cultivated olive in each province (Table 5.1, Figure 5.1). Soil samples were collected and analyzed as described by Archidona-Yuste *et al*. (2016c).

Nematodes were extracted from a 500-cm^3 sub-sample of soil by a modification of Cobb´s decanting and sieving method (Flegg 1966). Since recovery nematode effectiveness is highest in Cobb´s decanting and sieving method (Flegg 1966, Brown and Boag 1988), these data were used for prevalence and abundance data analyses. In some samples in which new taxa were detected and more specimens were required for suitable descriptions, additional soil samples were extracted by centrifugal-flotation (Coolen 1979). The nematode sample processing was carried out as described by Archidona-Yuste *et al*. (2016c). PPN from soil samples were identified to genus, and then we focussed on the species delineation of dagger nematodes of the genus *Xiphinema*.

2.3 Diversity indexes

Based on the *Xiphinema* spp. populations detected infesting soils from olives in Andalusia, conventional ecological and diversity indexes were performed in order to evaluate the distribution and changes in the diversity in wild and cultivated olives. In this regard, abundance and prevalence of each *Xiphinema* species identified were estimated. For each sampling site, abundance was calculated as the mean number of *Xiphinema* nematodes per 500 cm^3 of soil for all samples. The prevalence was computed by dividing the number of samples in which a *Xiphinema* species was detected by the total number of samples and expressed as a percentage.

Several diversity indexes including Hill´s diversity, Hill´s reciprocal of D (Simpson´s dominance index) and Hill´s evenness indexes (Hill 1973) were calculated according to code indications described by Neher and Darby (2009) using the SAS 9.4 software; in addition, Richness index was obtained using principal function implemented in the 'vegan' version 2.2–1 package (Oksanen *et al.* 2015) with the R version 3.1.1 software (R Core Development Team). Additionally, abundance and diversity indexes results were subjected to a univariate analysis of variance (ANOVA) and mean values were compared by the Tukey's test (Steel and Torrie 1980) for $P < 0.05$ using the general model procedure of SAS (Statistical Analysis System v. 9.4; SAS Institute, Cary, NC, USA).

2.4 Morphological studies

Xiphinema specimens for light microscopy were killed by gentle heat, fixed and examined *Xiphinema* specimens as described by Archidona-Yuste et al. and Seinhorst (Seinhorst 1962, Archidona-Yuste *et al.* 2016c). The morphometric study and drawing of each nematode population was carried out as described in previous papers (Loof and Luc 1990, Jairajpuri and Ahmad 1992, Lamberti *et al.* 2000, Coomans *et al.* 2001, Archidona-Yuste *et al.* 2016c). All abbreviations used are as defined in Jairajpuri and Ahmad (1992). In addition, a comparative morphological and morphometrical study of type specimens of some species were conducted with specimens kindly provided by Dr. A. Troccoli, from the nematode collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (*viz. Xiphinema cadavalense* Bravo and Roca 1995, and Dr. T. Mateille, from the French Nematode Collection, IRD, Montpellier, France (slides 15368–15376) (*viz. Xiphinema conurum* Siddiqi 1964. Nematode populations of *Xiphinema* species already described were analysed morphologically and molecularly in this study and proposed as standard and reference populations for each species given until topotype material becomes available and molecularly characterized. Voucher specimens of these described species have been deposited in the nematode collection of Institute for Sustainable Agriculture, IAS-CSIC, Córdoba, Spain.

Table 5.1 Taxa sampled for *Xiphinema* species and sequences used in this study.

Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
1. *X. andalusiense* sp. nov.	AR093	Belmez (Córdoba, Spain)	wild olive	KX244884	KX244921	KX244941
	AR093	Belmez (Córdoba, Spain)	wild olive	-	KX244922	-
	AN419	Andújar (Jaén, Spain)	wild olive	KX244885	KX244923	KX244942
	AN419	Andújar (Jaén, Spain)	wild olive	KX244886	KX244924	-
	AN419	Andújar (Jaén, Spain)	wild olive	KX244887	-	-
	AR108	Villaviciosa (Córdoba, Spain)	wild olive	KX244888	KX244925	-
2. *X. celtiense sp. nov.*	AR083	Peñaflor (Sevilla, Spain)	wild olive	KX244889	KX244926	KX244943
	AR082	Adamuz (Córdoba, Spain)	wild olive	KX244890	KX244927	-
3. *X. iznajarense sp. nov.*	JAO25	Iznájar (Córdoba, Spain)	culti. olive	KX244891	KX244928	KX244944
	JAO25	Iznájar (Córdoba, Spain)	culti. olive	KX244892	KX244929	-
4. *X. mengibarense* sp. nov.	OO3V4	Mengibar (Jaén, Spain)	culti. olive	KX244893	KX244930	KX244945
	OO3C5	Mengibar (Jaén, Spain)	culti. olive	KX244894	KX244931	-
	OO3C2	Mengibar (Jaén, Spain)	culti. olive	KX244895	-	-
5. *X. adenohystherum* Lamberti *et al*. 1992	AR063	Coto Ríos (Jaén, Spain)	wild olive	KX244896	-	-
	AR078	Almodóvar del Río (Córdoba, Spain)	wild olive	KX244897	-	-
	JAO06	La Granjuela (Córdoba, Spain)	culti. olive	KX244898	-	-
6. *X. baetica* Gutiérrez-Gutiérrez *et al.* 2013	AR088	Vejer de la Frontera (Cádiz, Spain)	wild olive	KX244899	-	-
7. *X. cadavalense* Bravo and Roca 1995	ST077	Espiel (Córdoba, Spain)	culti. olive	KX244900	KX244932	KX244946
8. *X. cohni* Lamberti *et al*. 1992	AR016	Sanlúcar de Barrameda (Cádiz, Spain)	wild olive	KX244901	KX244933	-
9. *X. conurum* Siddiqi 1964	ST045	Uleila del Campo (Almería, Spain)	culti. olive	KX244902	KX244934	KX244947
10. *X. coxi europaeum* Tarjan 1964	AR092	Alcolea (Córdoba, Spain)	wild olive	KX244903	-	-
	JAO04	Fuente Obejuna (Córdoba, Spain)	culti. olive	*	-	-
11. *X. duriense* Lamberti *et al.* 1993	ST002	Gibraleón (Huelva, Spain)	culti. olive	KX244904	KX244935	-

Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
	AR120	Paterna del Campo (Huelva, Spain)	wild olive	*	-	-
12. *X. hispanum* Lamberti *et al*. 1992	AR052	Andújar (Jaén, Spain)	wild olive	KX244905	-	-
13. *X. hispidum* Roca and Bravo 1994	AR004	Medina Sidonia (Cádiz, Spain)	wild olive	KX244906	-	-
	AR098	Almonte (Huelva, Spain)	wild olive	*	-	-
14. *X. incertum* Lamberti *et al.* 1983	AR030	Tarifa (Cádiz, Spain)	wild olive	KX244907	-	-
	AR020	Hinojos (Huelva, Spain)	wild olive	KX244908	-	-
	AR104	Mollina (Málaga, Spain)	wild olive	KX244909	-	-
	ST013	Osuna (Seville, Spain)	culti. olive	*	-	-
15. *X. index,* Thorne and Allen 1950	ST123	Adamuz (Córdoba, Spain)	culti. olive	KX244910	-	-
16. *X. italiae* Meyl 1953	AR021	Hinojos (Huelva, Spain)	wild olive	*	-	-
	AR041	Las Tres Villas (Almería, Spain)	wild olive	KX244911	KX244936	-
	AR118	Benahavis (Málaga, Spain)	wild olive	*	-	-
	AR091	Puerto Real (Cádiz, Spain)	wild olive	KX244912	KX244937	-
	ST079	Huévar del Aljarafe (Seville, Spain)	culti. olive	*	-	-
17. *X. lupini* Roca and Pereira 1993	AR099	El Rocío (Huelva, Spain)	wild olive	*	-	-
	AR110	Almadén de la Plata (Sevilla, Spain)	wild olive	*	-	-
18. *X. macrodora* Archidona-Yuste *et al*. 2016	JAO06	La Granjuela (Córdoba, Spain)	culti. olive	*	*	*
	JAO47	Santa Olalla del Cala (Huelva, Spain)	culti. olive	*	*	*
	AR097	Santa Mª de Trassierra (Córdoba, Spain)	wild olive	*	*	*
19. *X. madeirense* Brown *et al.* 1992	AR031	Tarifa (Cádiz, Spain)	wild olive	*	*	-
20. *X. nuragicum* Lamberti *et al*. 1992	JAO36	Casarabonela (Málaga, Spain)	culti. olive	KX244913	-	-
	AR055	San José del Valle (Cádiz, Spain)	wild olive	*	-	-
	JAO79	Úbeda (Jaén, Spain)	culti. olive	*	-	-
	JAO87	Pedro Martínez (Granada, Spain)	culti. olive	*	-	-
21. *X. oleae* Archidona-Yuste *et al*. 2016	AR035	Tarifa (Cádiz, Spain)	wild olive	*	*	*

Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
22. *X. opisthohysterum* Siddiqi 1961	AR031	Tarifa (Cádiz, Spain)	wild olive	*	KX244938	-
23. *X. pachtaicum* (Tulaganov, 1938) Kirjanova 1951	AR040	Riogordo (Málaga, Spain)	wild olive	*	-	-
	AR073	Castillo de Locubín (Jaén, Spain)	wild olive	*	-	-
	AR042	Tabernas (Almería, Spain)	wild olive	*	-	-
	JAO61	Paterna del Campo (Huelva, Spain)	culti. olive	*	-	-
24. *X. parapachydermum* Gutiérrez-Gutiérrez *et al*. 2013	AR035	Tarifa (Cádiz, Spain)	wild olive	KX244914	-	-
	ST122	Adamuz (Córdoba, Spain)	culti. olive	*	-	-
25. *X. plesiopachtaicum* Archidona-Yuste *et al*. 2016	AR063	Coto Ríos (Jaén, Spain)	wild olive	*	-	-
26. *X. pseudocoxi* Sturhan 1984	AR095	Alcaracejos (Córdoba, Spain)	wild olive	KX244915	KX244939	KX244948
	AR095	Alcaracejos (Córdoba, Spain)	wild olive	KX244916	KX244940	-
27. *X. santos* Lamberti *et al.* 1993	AR126	Arcos de la Frontera (Cádiz, Spain)	wild olive	*	-	-
28. *X. sphaerocephalum* Lamberti *et al*. 1992	AR073	Castillo de Locubín (Jaén, Spain)	wild olive	KX244917	-	-
29. *X. rivesi* Dalmasso 1969	ST076	Bollullos Par del Condado (Huelva, Spain)	culti. olive	*	-	-
30. *X. turcicum* Luc 1963	ST090	Santa Cruz del Comercio (Granada, Spain)	culti. olive	KX244918	-	-
	ST149	Prado del Rey (Cádiz, Spain)	culti. olive	KX244919	-	-
	ST199	Úbeda (Jaén, Spain)	culti. olive	*	-	-
	AR124	Sanlúcar la Mayor (Sevilla, Spain)	wild olive	*	-	-
	JAO39	Monda (Málaga, Spain)	culti. olive	*	-	-
31. *X. turdetanense* Gutiérrez-Gutiérrez *et al*. 2013	AR090	El Puerto de Sta. María (Cádiz, Spain)	wild olive	KX244920	-	-
	AR017	Sanlúcar de Barrameda (Cádiz, Spain)	wild olive	*	-	-
32. *X. vallense* Archidona-Yuste *et al*. 2016	AR055	San José del Valle (Cádiz, Spain)	wild olive	*	*	-
	AR027	Tarifa (Cádiz, Spain)	wild olive	*	-	-
	H0003	Hinojos (Huelva, Spain)	culti. olive	*	-	-

(-) Not obtained or not performed.
(*) Sequenced population but not deposited in GenBank database, since was identical to other sequences of the same specie

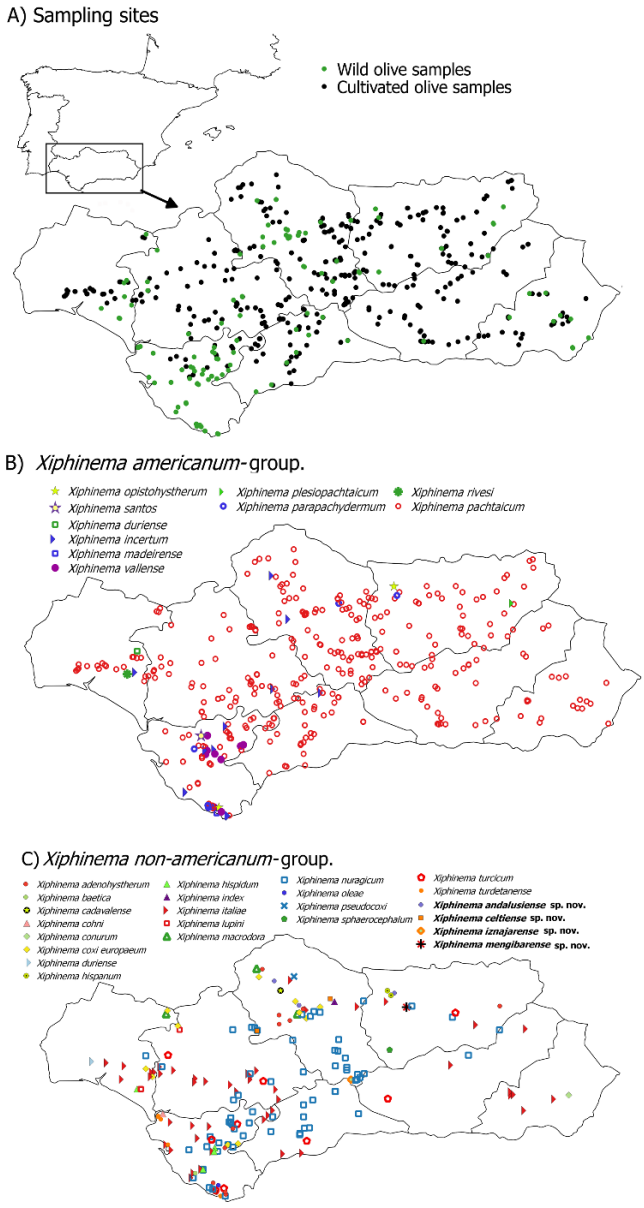

Figura 5.1: Geographic distribution of dagger nematodes of the genus *Xiphinema* in the present fieldworks on wild and cultivated olive in southern Spain. This map may be similar but not identical to other published maps of Andalusia and is therefore for illustrative purposes only on the sampling sites.

2.5 DNA extraction, PCR and sequencing

For molecular analyses, in order to avoid mistakes in the case of mixed populations, two live nematodes from each sample were temporary mounted in a drop of 1M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed to the unidentified populations of *Xiphinema*. Following morphological confirmation, the specimens were removed from the slides and DNA extracted.

Detailed protocols for nematode DNA extraction, PCR and sequencing were applied as described by Castillo *et al*. (2003). The D2-D3 expansion segments of 28S rRNA, ITS1 region, and the portion of the 18S-rRNA were amplified using primers described in previous papers (Vrain *et al.* 1992, Cherry *et al.* 1997, Ley *et al.* 1999, Holterman *et al.* 2006, Archidona-Yuste *et al.* 2016c). PCR products were purified and sequenced as described by Archidona-Yuste *et al*. (2016c). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 5.1.

2.6 Phylogenetic analysis

D2-D3 expansion segments of 28S rRNA, ITS1, and partial 18S rRNA sequences of different *Xiphinema* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (Gutiérrez-Gutiérrez *et al.* 2013b, Archidona-Yuste *et al.* 2016b, c). Multiple alignments of the different genes were made using the Q-INS-i algorithm of MAFFT v. 7.205 (Katoh and Standley 2013), strategy FFT-NS-1 with default parameters. Sequence alignments were visualized using BioEdit (Hall 1999) and edited by Gblocks v0.91b (Castresana 2000) in Castresana Lab server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using the less stringent option (Minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences + 1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Percentage similarity between sequences was calculated using a sequence identity matrix in BioEdit. For that, the

score for each pair of sequences was compared directly and all gap or place-holding characters were treated as a gap. When position of both sequences has a gap they do not contribute as a difference. Phylogenetic analyses of the sequence data sets were performed based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best fitted model of DNA evolution was obtained using jModelTest v. 2.1.7 (Darriba *et al.* 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analyses were performed under GTR+I+G (namely, general time reversible of invariable sites and a gamma-shaped distribution) model for D2-D3 expansion segments of 28S and ITS1 rRNA, and TIM3+I+G (namely, transversional and a transitional of invariable sites and a gamma-shaped distribution) model for the partial 18 S rDNA. These BI analyses were run separately per dataset using four chains for 2 × 106 generations, respectively. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996).

2.7 Nomenclatural Acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature (ICZN), and hence the new names contained herein are available under that Code from the electronic edition. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: urn:lsid:zoobank.org:pub:CE945C7D-7B14-46DD-8A17-A93A05750590. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS.

3. Results

3.1 Taxon sampling, abundance, prevalence and diversity indexes of *Xiphinema* spp. in olive

All *Xiphinema* spp. found in this study including specimens of sampling sites used in morphological and/or molecular analyses, are shown in Table 5.1. In addition, all positive *Xiphinema* spp. and sampling sites are presented in Figure 5.1. Overall, 32 *Xiphinema* spp. were detected in the rhizosphere of olive trees, ten species belonging to *X. americanum*-group, whereas 22 were attributed to *X.* non-*americanum*-group (Table 5.2). From all *Xiphinema* spp. identified in this study, 26 species were associated with wild olive, whereas seventeen *Xiphinema* species were associated with cultivated olive (Table 5.1, Figure 5.1). Eleven *Xiphinema* species occurred in both wild and cultivated olives (*viz. X. adenohystherum* Lamberti *et al*. 1992, *X. coxi europaeum* Tarjan 1964, *X. duriense* Lamberti *et al*. 1993, *X. incertum* Lamberti *et al*. 1983, *X. italiae* Meyl 1953, *X. macrodora*, *X. nuragicum* Lamberti *et al.* 1992, *X. pachtaicum* (Tulaganov, 1938) Kirjanova 1951, *X. parachydermum* Gutiérrez-Gutiérrez *et al*. 2012, *X. turcicum* Luc 1963 and *X. vallense*), while the remaining 21 identified species where present either in wild or cultivated olives only.

Xiphinema spp. were present in low to high densities (*ca* 33, from 1 to 414 nematodes per 500 cm^3 of soil) in both wild and cultivated olives, being observed in cultivated olives in higher densities than in wild olives (Tables 5.2 and S5.10). Nematode abundance of *X. americanum*-group species was significantly higher ($P < 0.01$) in cultivated than wild olives (Figure 5.2B), averaging *ca* 23 vs 43 nematodes per 500 cm^3 of soil for wild and cultivated olives, respectively. On the contrary, nematode density was similar ($P > 0.05$) in both olive types in the *Xiphinema* non-*americanum*-group Figure 5.2C), being slightly higher in wild than cultivated olives. In general, *Xiphinema* spp. belonging to *X. americanum*-group showed higher densities than species identified within *X.* non-*americanum*-group (*ca* 38 vs 22 nematodes per 500 cm^3 of soil, respectively) (Tables 5.2 and S5.10), which resulted in a higher abundance ($P < 0.001$) for *X. americanum*-group than *X.* non-*americanum*-group species (Figure 5.2D). On the other hand,

the *Xiphinema* species with the highest nematode density was *X. pachtaicum* (414 nematodes per 500 cm^3 of soil), which showed a higher average density in cultivated than wild olives (Tables 5.2 and S5.10). However, the subsequent species with high nematode density included *X. italiae* and *X. nuragicum* (350 and 218 nematodes per 500 cm^3 of soil, respectively), both belonging to *X.* non-*americanum*-group, showing lower average density in cultivated than in wild olives (Tables 5.2 and S5.10).

The overall prevalence of *Xiphinema* spp. in olive was 85.0% (385 out of 453 sample sites) in Andalusia Figure 5.1, Tables 5.2 and S5.10). However, *Xiphinema* spp. were more prevalent in wild olives (93.9%, 108 out of 115 sampling sites) than cultivated olives (81.7%, 276 out of 338 sampling sites) (Table 5.2). In addition, the major differences between both olive types occurred in the *Xiphinema* non-*americanum*-group species, being more prevalent in wild than cultivated olives. Nevertheless, prevalence in *X. americanum*-group species was similar between both olive types (Table 5.2). As indicated above for most of the *Xiphinema* spp. identified in this study, the prevalence was higher in wild than cultivated olive except for *X. pachtaicum* that was detected in both wild and cultivated olives in all provinces of Andalusia, and being the most prevalent *Xiphinema* species in our study (74.2%, 336 out of 453 sample sites) (Tables 5.2 and S5.10). The subsequent species with a high prevalence was *X. nuragicum* (16.3%, 74 out of 453 sample sites) that was detected in both olive types in the most of the Andalusia provinces, at exception of Almería (Figure 5.1, Tables 5.2 and S5.10). Another prevalent *Xiphinema* species belonging also to *X.* non-*americanum*-group was *X. italiae* (10.2%, 46 out of 453 sample sites), that was found in both olive types in Almería, Cádiz, Huelva and Málaga provinces, but only in wild olive in Córdoba, Granada, Jaén and Seville provinces (Figure 5.1, Tables 5.2 and S5.10).

Table 5.2 Soil nematode population density (number of specimens) and prevalence (%) of *Xiphinema* spp. in wild and cultivated olives in Andalusia, southern Spain.

Host plant [a]	**Wild olive (W)**				**Cultivated olive (C)**				**Global data (W + C)**			
Number of samples	**115**				**338**				**453**			
	Density[b]	**Min**[b]	**Max**[b]	**Prevalence**[c]	**Density**	**Min**	**Max**	**Prevalence**	**Density**	**Min**	**Max**	**Prevalence**
***Xiphinema* spp.**	**22.8 ± 35.8**	**1**	**350**	**93.9**	**38.1 ± 53.6**	**1**	**414**	**81.7**	**32.6 ± 48.6**	**1**	**414**	**85.0**
***X. americanum*-group spp.** [d]	**22.6 ± 23.7**	**1**	**116**	**78.3**	**43.4 ± 57.8**	**1**	**414**	**79.9**	**37.9 ± 51.9**	**1**	**414**	**79.7**
Xiphinema duriense	2 ± 0	2	2	0.90	1 ± 0	1	1	0.30	1.3 ± 0.6	1	2	0.44
Xiphinema incertum	22.9 ± 11.3	1	42	9.60	38 ± 0	38	38	0.30	24.2 ± 11.6	1	42	4.74
Xiphinema madeirense	11 ± 0	11	11	0.90	-	-	-	-	11 ± 0	11	11	0.22
Xiphinema opisthohysterum	8.5 ± 7.8	3	14	1.70	-	-	-	-	8.5 ± 7.8	3	14	0.44
Xiphinema pachtaicum	22.7 ± 25.0	1	116	58.3	43.9 ± 58.3	1	414	79.4	39.7 ± 54.0	1	414	74.2
Xiphinema parapachydermum	28.6 ± 7.8	16	34	4.30	8 ± 0	8	8	0.30	25.2 ± 10.9	8	34	1.32
Xiphinema plesiopachtaicum	112 ± 0	112	112	0.90	-	-	-	-	112 ± 0	112	112	0.22
Xiphinema santos	9 ± 0	9	9	0.90	-	-	-	-	9 ± 0	9	9	0.22
Xiphinema rivesi	-	-	-	-	58 ± 0	58	58	0.30	58 ± 0	58	58	0.22
Xiphinema vallense	13.6 ± 12.8	2	37	6.10	14.0 ± 2.9	12	16	0.60	13.7 ± 11.1	2	37	1.99
X. non-americanum-group spp. [d]	**23.1 ± 44.5**	**1**	**350**	**70.4**	**21.2 ± 32.2**	**1**	**218**	**25.1**	**22.2 ± 39.2**	**1**	**350**	**36.6**
***Xiphinema andalusiense* sp. nov.**	13.7 ± 8.7	4	21	2.6	-	-	-	-	13.7 ± 8.7	4	21	0.66
***Xiphinema celtiense sp.* nov.**	42.5 ± 55.9	3	82	1.7	-	-	-	-	42.5 ± 55.9	3	82	0.44
***Xiphinema iznajarense sp.* nov.**	-	-	-	-	34 ± 0	34	34	0.30	34 ± 0	34	34	0.22
***Xiphinema mengibarense* sp. nov.**	-	-	-	-	12 ± 0	12	12	0.30	12 ± 0	12	12	0.22
Xiphinema adenohystherum	6.2 ± 4.9	1	14	11.3	1 ± 0	1	1	0.30	5.9 ± 4.9	1	14	3.09

Host plant [a]	Wild olive (W)				Cultivated olive (C)				Global data (W + C)			
	Density[b]	Min[b]	Max[b]	Prevalence[c]	Density	Min	Max	Prevalence	Density	Min	Max	Prevalence
Xiphinema baetica	1 ± 0	1	1	0.90	-	-	-	-	1 ± 0	1	1	0.22
Xiphinema cadavalense	-	-	-	-	1 ± 0	1	1	0.30	1 ± 0	1	1	0.22
Xiphinema cohni	32 ± 0	32	32	0.90	-	-	-	-	32 ± 0	32	32	0.22
Xiphinema conurum	-	-	-	-	3 ± 0	3	3	0.30	3 ± 0	3	3	0.22
Xiphinema coxi europaeum	14.3 ± 28.0	1	88	7.80	1 ± 0	1	1	0.60	11.9 ± 25.6	1	88	2.43
Xiphinema hispanum	6.5 ± 7.8	1	12	1.7	-	-	-	-	6.5 ± 7.8	1	12	0.44
Xiphinema hispidum	6.6 ± 5.9	1	14	4.30	-	-	-	-	6.6 ± 5.9	1	14	1.10
Xiphinema index	-	-	-	-	3 ± 0	3	3	0.30	3 ± 0	3	3	0.22
Xiphinema italiae	45.9 ± 97.4	3	350	11.3	20.8 ± 27.1	1	121	9.70	27.6 ± 55.5	1	350	10.2
Xiphinema lupini	6.7 ± 4.6	4	12	2.60	-	-	-	-	6.7 ± 4.6	4	12	0.66
Xiphinema macrodora	7 ± 0	7	7	0.90	11.0 ± 4.2	8	14	0.60	9.7 ± 3.8	7	14	0.66
Xiphinema nuragicum	34.5 ± 37.6	1	134	31.3	26.9 ± 40.5	1	218	11.2	30.7 ± 39.1	1	218	16.3
Xiphinema oleae	4 ± 0	4	4	0.90	-	-	-	-	4 ± 0	4	4	0.22
Xiphinema pseudocoxi	10 ± 0	10	10	0.90	-	-	-	-	10 ± 0	10	10	0.22
Xiphinema sphaerocephalum	15 ± 0	15	15	0.90	-	-	-	-	15 ± 0	15	15	0.22
Xiphinema turcicum	2.3 ± 1.3	1	4	1.70	9.4 ± 8.9	1	22	1.50	6.2 ± 7.3	1	22	1.55
Xiphinema turdetanense	2.2 ± 1.3	1	4	4.30	-	-	-	-	2.2 ± 1.3	1	4	1.55

[a] Host plant: W = wild olive; C = cultivated olive.

[b] Population density was calculated as the mean of *Xiphinema* nematodes per 500 cm^3 of soil. Average, standard deviation, minimum and maximum levels in fields/host where this genus, group species or species were detected.

[c] The prevalence was computed by dividing the numbers of samples in which the *Xiphinema* species was observed by the total number of samples and expressed as a percentage

[d] *Xiphinema* group species established by Tarjan (1964); Loof and Luc (1972); Lamberti *et al*. (2000); and Coomans *et al*. (2001)

(-) not found

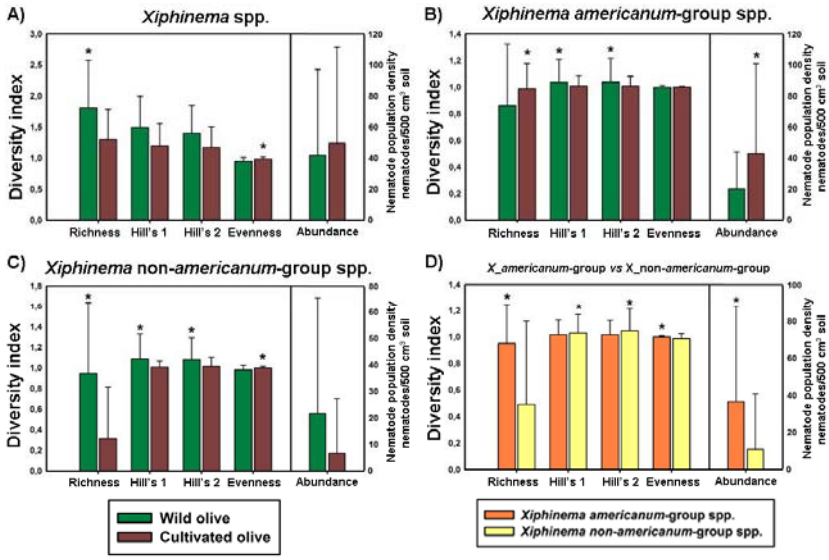

Figura 5.2: Summary barplot of nematode abundance, Richness, Hill´s diversity (Hill´s 1), Hill´s reciprocal of D (Simpson´s dominance index) (Hill´s 2) and Hill´s evenness diversity indexes derived from results of *Xiphinema* spp. identification in 385 sampling sites of olives orchards (Figure 1) grouped by olive type (wild and cultivated olive) and *X*. *americanum*-group and *X*. non-*americanum*-group species. Error bars indicate the standard error of the mean. Significance = F probability of main effects in ANOVA, according to Tukey´s test (Steel and Torrie, 1980) for $P < 0.05$.

Several diversity indexes were estimated in our study (Richness, Hill´s diversity, Hill´s reciprocal of D (Simpson´s dominance index), and Hill´s evenness (Hill 1973)), and tested for differences associated with presence of *Xiphinema* spp. in wild and cultivated olive (Figure 5.2). Overall, the number of *Xiphinema* spp. detected in each sampling site (Richness index) was significantly affected ($P < 0.05$) by olive type (Figure 5.2), showing higher values ($P < 0.001$) in wild than cultivated olives (Figure 5.2A). Similarly, Richness index in *X.* non-*americanum*-group species were significantly higher ($P < 0.05$) in wild than in cultivated olive (Figure 5.2C), but the opposite occurred in the *X. americanum*-group species (Figure 5.2B). Overall, the Richness index was significantly higher ($P < 0.001$) in *X. americanum*-group than in *X.* non-*americanum*-group (Figure 5.2D). Diversity and dominance diversity indexes (Hill´s 1 and Hill´s 2,

respectively) showed similar patterns for both olive types (Figure 5.2). Thus, significant differences ($P < 0.05$) for both diversity indexes were observed when *Xiphinema* species groups were considered separately (Figure 5.2B, C). On the other hand, the detection of a higher number of species belonging to *X.* non-*americanum*-group linked to the increased presence of prevalent species (*viz. X. italiae*, *X. nuragicum* or *X. coxi europaeum*) than *X. americanum*-group (Tables 5.1 and S5.10) resulted in significant differences ($P < 0.01$) among them when it was considered both olive types (Figure 5.2D). Evenness diversity showed an inverse trend to that observed in diversity and dominance diversity indexes, with cultivated olives showing higher values ($P < 0.01$) than that of wild olives (Figres 5.2A, C) according to the higher abundance and prevalence ($P < 0.05$) detected in cultivated than wild olives (Tables 5.2 and S5.10). On the other hand, Evenness index in *X. americanum*-group was significantly higher ($P < 0.001$) than that of *X.* non-*americanum*-group species (Figure 5.2D).

3.2 Taxonomic treatment

Nematoda Linnaeus, 1758
Dorylaimida Pearse, 1942
Longidoridae Thorne, 1935
Longidorinae Thorne, 1935
Xiphinema Cobb, 1913

3.2.1 *Xiphinema andalusiense sp. nov.*

urn:lsid:zoobank.org:act:95E9BE47-B822-4AAF-A11C-50EF7A016137

Holotype

Adult female, collected from the rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) (38°15'10.3"N, 005°09'53.3"W), at Belmez, Córdoba province, Spain; collected by G. Leon Ropero, March 14, 2015; mounted in pure glycerine and deposited in the nematode collection at

Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR093-2).

Paratypes

Female and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers AR093-5-AR093-7); two females at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (AR093-8); and one female at USDA Nematode Collection, Beltsville, MD, USA (T-6774p); collected by G. Leon Ropero, March 14, 2015.

Diagnosis

Xiphinema andalusiense sp. nov. is an apparently parthenogenetic species belonging to morphospecies Group 5 from the *Xiphinema* non-*americanum*-group species (Loof and Luc 1990). It is characterized by a moderate long body (4.3–6.1mm), assuming an open C-shaped when heat-relaxed; lip region hemispherical almost continuous or separate from the body contour by a slightly depression, 12.5–15.5 µm wide; a 137.0–151.0 µm long odontostyle; vulva slightly anterior to middle of the body; reproductive system didelphic-amphidelphic with both branches about equally developed having a Z-differentiation in uterus in the form of 11–16 globular bodies in the vicinity of the *pars dilatata uteri*, and small spiniform structures and crystalloid bodies in low number; female tail short, convex-conoid to conical shape with distinctly digitate terminus, and bearing three pairs of caudal pores; c´ ratio (1.0–1.3); and specific D2-D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KX244884-KX244888, KX244921-KX244925, and KX244941-KX244942, respectively). According to the polytomous key of Loof and Luc (1990), the new species has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2+3-C5a-D5(6)-E5-F5(4)-G3-H2-I3-J4-K6-I1.

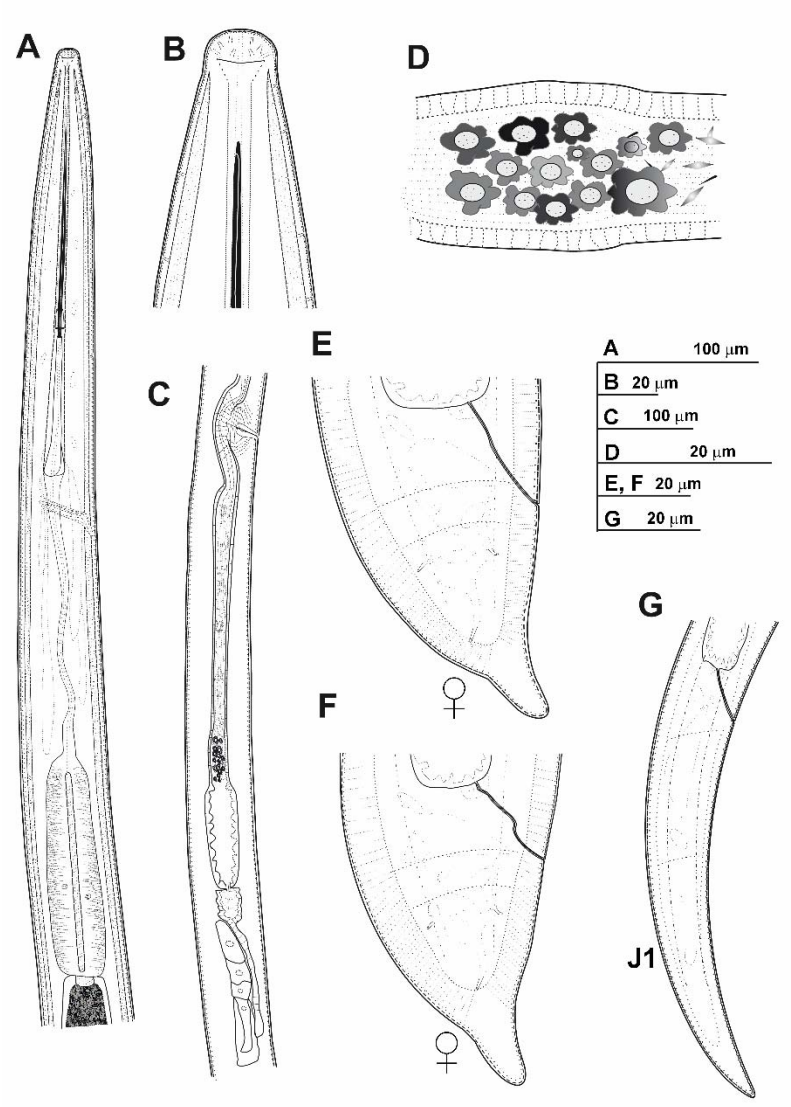

Figura 5.3: Line drawings of *Xiphinema andalusiense* sp. nov. female paratypes and first-stage juvenile A) Pharyngeal region. B) Detail of lip region. C) Posterior female genital branch showing Z-differentiation. D) Detail of Z-differentiation. E-F) Female tails. G) First-stage juvenile tail (J1).

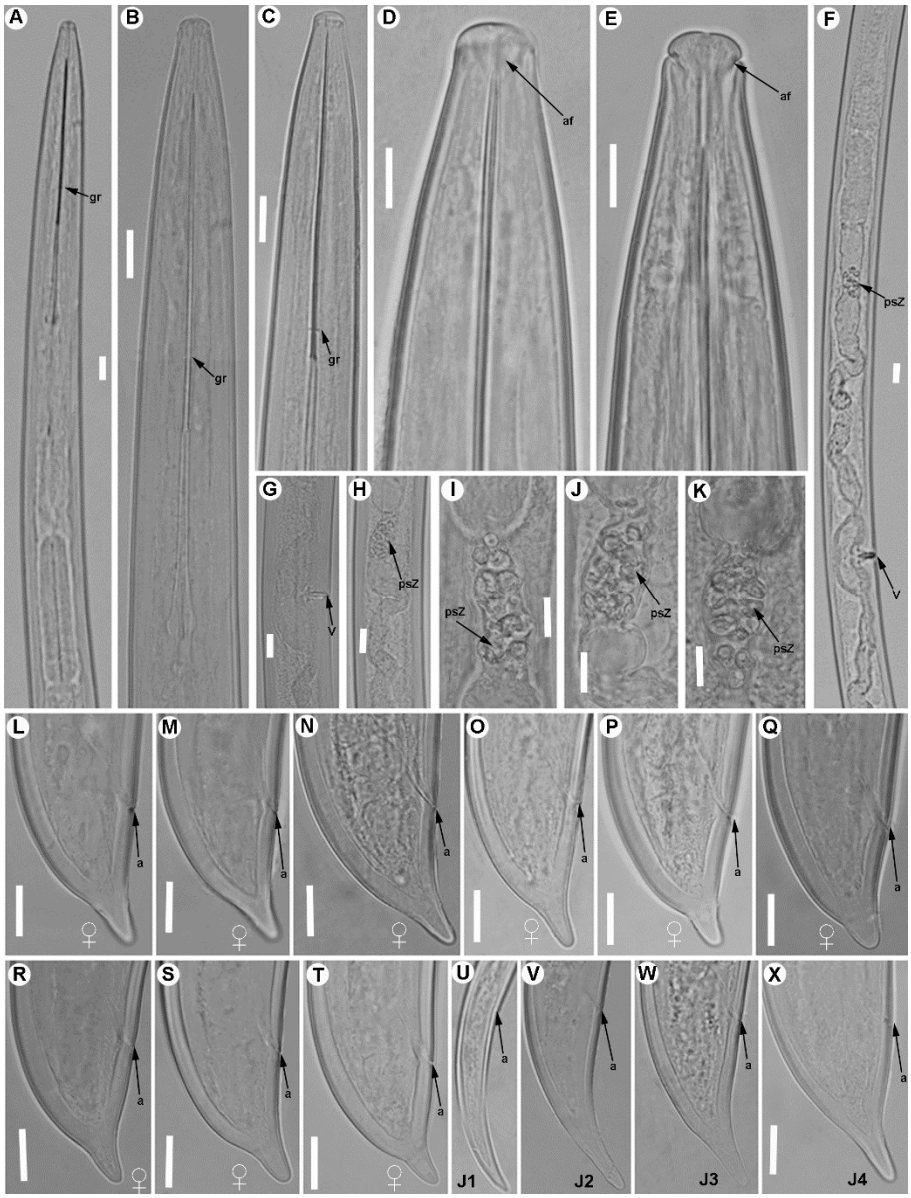

Figura 5.4: Light micrographs of *Xiphinema andalusiense* sp. nov. female paratypes and juvenile stages A) Pharyngeal region. B±E) Female anterior regions. F) Detail of anterior female gonad showing Z-differentiation. G) Vulval region. H) Detail of female genital track showing Z-differentiation. I-K) Z-differentiation. L-T) Female tails. U-X) First-, second-, third-, and fourth-stage juvenile (J1-J4) tails, respectively. Abbreviations: a = anus; cb = crystalloid bodies; gr = guiding-ring; odt = odontostyle; rodt = replacement odontostyle; spi = spiniform structures; spZ = Z-differentiation; v = vulva. Scale bars = 20 µm.

Etymology

The species epithet refers to the autonomous community from Spain, Andalusia, where the species was detected and moderately distributed.

Description of taxa

Female:

Body cylindrical and habitus in specimens killed by gentle heat as open C-shape, more curved behind the vulva position, with increasing curvature towards the posterior extremity. Cuticle 3.5–4.0 µm thick at mid-body, but thicker at tail tip, 4.5–8.0 µm wide. Lateral hypodermical chords 18.0–29.0 µm wide at mid body or 29–57% of the corresponding maximum body diameter. Lip region hemispherical, rounded laterally and less so frontally, almost continuous or separated from the body contour by a slightly depression, 12.5–15.5 µm diam. and 5.0–7.5 µm high. Amphidial fovea aperture extending for *ca* 76–88% of lip region diam. and located at *ca* two-thirds of lip region height. Odontostyle long, 1.6–1.9 times longer than odontophore, and the latter with moderate-developed flanges 9.5–12.5 µm wide. Guiding ring with average guiding sheath length of 16.0 µm. Pharynx occupying about 8–15% of body length, consisting of an anterior slender narrow part 346–541 µm long and extending to terminal pharyngeal bulb occupying *ca* 19–27% of total pharyngeal length, 112–139 µm long and 22.5–29.5 µm wide. Glandularium 99.5–119.0 µm long. Nucleus of dorsal pharyngeal gland (DN) located at beginning of basal bulb (10.4–14.3%), ventrosublateral nuclei (SVN) situated *ca* halfway along bulb (46.9–59.4%) (position of gland nuclei calculated as described by Loof and Coomans (1972). In some specimens studied the tip of reserve odontostyle (vestigium) was *ca* 3.5 5 µm in size and directed anteriorly to the isthmus. Cardia conoid, 6.5–14.5 µm long. Prerectumvariable in length, 372–783 µm long or 10–19 times anal body diam. Rectum 35.5–47.0 µm long ending in anus as a small rounded slit. Reproductive system didelphic-amphidelphic with branches equally developed and vulva slit-like situated located slightly anterior to mid body. Each branch composed of a 109–212 µm long reflexed ovary and a largely tubular oviduct with enlarged *pars dilatata oviductus* separated from uterus by a well developed sphincter. Uterus tripartite, comprising a developed *pars dilatata uteri* continuing into a narrower, muscular tube-like portion including a Z-differentiation with

weakly muscularized wall and containing 11–16 globular bodies of variable size, each one consisting of a large central portion, irregularly spherical surrounded by a variable number of refractive pieces, and petal shaped (Figures 5.3 and 5.4). Low numbers of small spiniform structures and crystalloid bodies along uterus, observed in fresh material in water. Abundant wrinkles observed in uterine wall along uterus, which may be confused as spiniform structures. No sperm was observed in the female genital tract. Ovejector well-developed 41.5–60.0 µm wide, and vagina perpendicular to body axis, 19.5–33.5 µm long or 27–52% of corresponding body diam. in lateral view. In some specimens studied, maturate eggs observed in the tubular part of uterus, 156–183 µm long and 35–43 µm wide. Tail short, varying from convex-conoid to conoid shape with digitate or subdigitate terminus, directed ventrally with respect to the body axis. Distinct terminal blind canal, and in most of specimens studied three caudal pores present on each side.

Male:

Not detected.

Description of juveniles:

All four juvenile stages (first-, second-, third- and fourth-stage) were identified Using morphological characters such as body length, length of replacement and functional odontostyle (Table 5.3, Figure 5.5) (Robbins *et al.* 1995, 1996). Specifically, J1 were characterised by position of replacement odontostyle just posterior to functional odontostyle, its tip touching or very close to base of functional odontostyle; tail elongate conoid with a slightly dorsal depression at hyaline region and c' ratio ≥ 3.5 (Figures 5.3 and 5.4); and odontostyle length *ca* 66 µm. Tail morphology in second-juvenile stage similar to J1, becoming shorter and stouter than this developmental stage. However, tail morphology in third- and fourth-juvenile stages (except for undeveloped genital structures) similar to that of female, including almost conoid tail shape ending in a digitate terminus (Figure 5.4), becoming progressively shorter and stouter in each moult, and shorter distance from anterior end to guiding-ring in each moult.

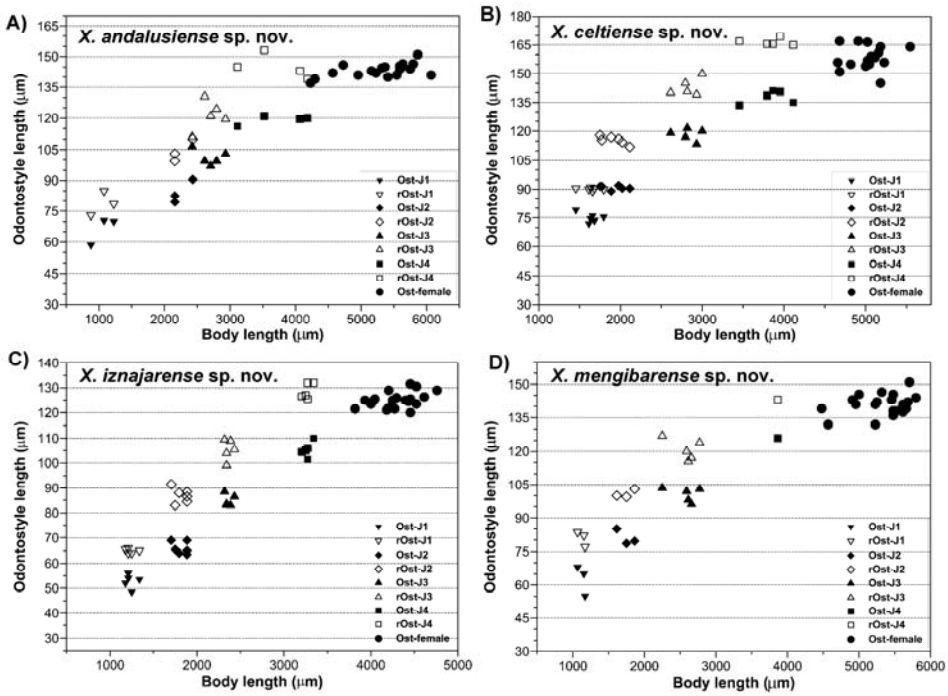

Figura 5.5: Relationship between body length and functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from first-stage juveniles (J1) to mature females of: A) *Xiphinema andalusiense* sp. nov. B) *Xiphinema celtiense* sp. nov. C) *Xiphinema iznajarense* sp. nov. D) *Xiphinema mengibarense* sp. nov.

Measurements, morphology and distribution:

Morphometric variability is described in Tables 5.3 and 5.4 and morphological traits in Figures 5.3-5.4. In addition to the type locality, *Xiphinema andalusiense* sp. nov. was collected from the rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) of two localities belonging to Córdoba and Jaén provinces, being one of the new species described in this work which has a broader distribution in Andalusia, concretely on North of Andalusia (Table 5.1, Figure 5.1).

Table 5.3 Morphometrics of females and juvenile developmental stages of *Xiphinema andalusiense* sp. nov. from the rhizosphere of wild olive at Belmez (Córdoba province) southern Spain[a].

Host/locality, sample code	**wild olive, Belmez (Córdoba province) AR093**					
Characters/ratios[b]	**Holotype**	**Paratype Females**	**J1**	**J2**	**J3**	**J4**
n		19	4	3	6	4
L (mm)	5.4	5.3 ± 0.53	1.15 ± 0.23	2.25 ± 0.16	2.72 ± 0.18	3.72 ± 0.49
		(4.2-6.1)	(0.88-1.41)	(2.16-2.43)	(2.42-2.93)	(3.11-4.18)
a	84.5	80.3 ± 5.7	52.4 ± 7.1	57.6 ± 3.4	63.3 ± 5.3	73.0 ± 1.6
		(68.7-89.3)	(47.8-62.9)	(54.0-60.8)	(57.9-71.6)	(71.5-75.0)
b	9.5	10.0 ± 1.3	4.4 ± 0.7	7.5 ± 0.6	7.3 ± 0.5	7.9 ± 1.8
		(6.8-11.9)	(4.0-5.5)	(6.8-7.9)	(6.3-7.7)	(5.7-9.9)
c	127.3	112.9 ± 11.8	16.0 ± 2.1	31.7 ± 4.9	39.8 ± 6.3	66.1 ± 12.6
		(83.7-127.5)	(13.6-18.5)	(27.2-36.8)	(29.5-47.7)	(50.6-81.2)
c´	1.0	1.2 ± 0.1	4.7 ± 0.9	2.6 ± 0.4	2.2 ± 0.3	1.5 ± 0.2
		(1.0-1.3)	(3.5-5.5)	(2.2-3.0)	(1.9-2.7)	(1.4-1.8)
V	48.0	47.9 ± 1.2	-	-	-	-
		(46.0-50.5)	-	-	-	-
Odontostyle	140.0	143.4 ± 3.3	66.0 ± 5.6	84.2 ± 5.7	100.3 ± 3.8	119.1 ± 2.2
		(137.0-151.0)	(58.5-70.5)	(79.5-90.5)	(96.5-106.5)	(116.0-121.0)
Odontophore	86.5	82.3 ± 2.9	39.0 ± 4.4	49.3 ± 1.8	66.9 ± 3.5	76.8 ± 1.0
		(76.0-88.5)	(34.5-44.0)	(48.0-50.5)	(64.0-72.0)	(75.5-78.0)

Host/locality, sample code	wild olive, Belmez (Córdoba province) AR093					
Characters/ratios[b]	**Holotype**	**Paratype Females**	**J1**	**J2**	**J3**	**J4**
Total stylet	226.5	225.7 ± 5.1	-	-	-	-
		(217.5-239.5)	-	-	-	-
Replacement odontostyle	-	-	78.8 ± 4.9	104.2 ± 5.3	120.2 ± 7.0	145.0 ± 5.9
	-	-	(73.0-85.0)	(99.5-110.0)	(111.0-130.5)	(139.0-153.0)
Lip region diam.	12.5	13.4 ± 1.0	8.1 ± 0.5	9.2 ± 0.6	9.8 ± 1.1	11.5 ± 0.0
		(12.5-15.5)	(7.5-8.5)	(8.5-9.5)	(8.5-11.5)	(11.5-11.5)
Oral aperture-guiding ring	138.5	137.3 ± 7.7	48.9 ± 4.4	59.2 ± 7.3	80.8 ± 7.6	108.8 ± 7.4
		(119.5-148.0)	(44.0-53.5)	(51.0-65.0)	(67.5-88.0)	(102.0-118.5)
Tail length	42.5	47.0 ± 2.4	71.9 ± 10.1	71.7 ± 7.0	69.5 ± 8.0	57.0 ± 6.4
		(42.5-52.0)	(57.0-79.5)	(66.0-79.5)	(60.0-82.0)	(51.5-63.5)
J	14.0	18.6 ± 2.2	10.5 ± 1.3	20.2 ± 6.3	18.8 ± 1.7	21.1 ± 1.8
		(14.0-23.5)	(9.0-11.5)	(14.0-26.5)	(16.5-20.5)	(18.5-22.5)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; J (hyaline tail region length

Table 5.4 Morphometrics of females of *Xiphinema andalusiense* sp. nov. from the rhizosphere of wild olive at several localities (Córdoba and Jaén provinces) southern Spain[a].

Host/locality, sample code	wild olive, Villaviciosa (Córdoba province) AR108	wild olive, Andújar (Jaén province) AN419
Characters/ratios[b]	females	females
n	1	6
L (mm)	4.01	4.72 ± 0.37 (4.27-5.14)
a	64.4	84.9 ± 9.8 (73.5-97.8)
b	10.2	9.6 ± 1.1 (7.9-10.8)
c	83.8	97.1 ± 5.5 (90.9-105.9)
c´	1.2	1.3 ± 0.1 (1.2-1.3)
V	43.5	- -
Odontostyle	135.0	141.7 ± 4.4 (137.0-148.0)
Odontophore	70.0	79.9 ± 4.7 (71.0-84.0)
Total stylet	205.0	- -
Lip region diam.	11.5	13.5 ± 0.8 (12.0-14.5)
Oral aperture-guiding ring	124.0	132.5 ± 4.2 (129.5-141.0)
Tail length	48.0	48.6 ± 2.7 (46.0-53.5)
J	15.5	17.3 ± 1.1 (15.5-18.5)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; J (hyaline tail region length).

Relationships:

According to the polytomous key by Loof and Luc (1990) and sorting on matrix codes A (type of female genital apparatus), C (tail shape), D (c´ ratio), E (vulva position), F (body length), and G [total spear length (odontostyle + odontophore)], *X. andalusiense* sp. nov. closely resembles *X. baetica* Gutiérrez-Gutiérrez *et al*. 2013, *X. cadavalense*, and *X. turdetanense* Gutiérrez-Gutiérrez *et al*. 2013. *Xiphinema andalusiense* sp. nov. differs from *X. baetica* in few morphological characters including lower a ratio (64.4–89.3 vs 91.6–131.2), slightly lower c´ ratio (1.0–1.3 vs 1.1–1.8), the presence of spiniform structures or crystalloid bodies along tubular portion of uterus vs absent, and the absence vs presence of males (Gutiérrez-Gutiérrez *et al.* 2013b). On the other hand, *X. andalusiense* sp. nov. mainly differs from *X. cadavalense* in having a shorter odontostyle and odontophore length (135.0–151.0, 70.0–88.5 vs 150.5–164.5 µm, 90.0–111.5 µm, respectively) resulting in a shorter stylet length (215.5–239.5 vs 244.5–278.5 µm), a narrower lip region (12.0–15.5 vs 14.0–19.5 µm), and higher a and c´ ratios (64.4–89.3, 1.0–1.3 vs 454.5–70.9, 0.8–1.2, respectively) (Bravo and Roca 1995). Finally, *X. andalusiense* sp. nov. differs from *X. turdetanense* in having a slightly longer odontostyle length (137.0–151.0 vs 121.0–142.0 µm), a slightly narrower lip region (11.5–15.5 vs 14.0–16.0 µm), higher number of globular bodies present in the Z-differentiation (11–16 vs 6–8), size and number of spiniform structures presents along tubular part of uterus (low number and smaller vs high number and larger), presence of crystalloid bodies along uterus vs absence, and the absence vs presence of males (Gutiérrez-Gutiérrez *et al.* 2013b).

In addition, *X. andalusiense* sp. nov. is molecularly related to *X. macrodora*, but it can be clearly differentiated in having a smaller nematode body and odontostyle length (4.0–6.1mm, 137.0–151.0 µm vs 7.2–8.7mm, 190.0–206.0 µm, respectively) (Archidona-Yuste *et al.* 2016b).

Molecular divergence of the new species:

D2-D3 region of *X. andalusiense* sp. nov. (KX244884-KX244888) was 97% similar to *X. baetica* (KC567167, KX244899), *X. macrodora* (KU171040, KU171042) and X. cadavalense (KX244900); sequence variation among these species was from 24 to 34 nucleotides and from 3 to 8 indels (Table

5.5). *Xiphinema andalusiense* sp. nov. showed an intraspecific variation from 0 to 8 nucleotides and no indels. The closest species to *X. andalusiense* sp. nov. (KX244921-KX244925) in relation to the ITS1 region were also *X. baetica* (KC567156, 89% similar, 119 nucleotides and 28 indels), *X. cadavalense* (88% similar, 127 nucleotides and 34 indels), and *X. macrodora* (85% similar, 162 nucleotides and 61 indels). Intraspecific variation for this marker was 44 nucleotides and 23 gaps amongst the five studied populations (Table 5.5). Finally, the partial 18S region of *X. andalusiense* sp. nov. showed high similarity values (99%), with several *Xiphinema* spp. such as *X. baetica* (KC567148-KC567149), *X. cadavalense* (KX244932), *X. macrodora* (KU171050) and *X. coxi europaeum* (KC567153).

3.2.2 *Xiphinema celtiense* sp. nov.

urn:lsid:zoobank.org:act:17E565E4-18E8-4D60-AA57-55253F3E257E

Holotype

Adult female, collected from the rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) (38°02'50.9"N, 004°32'52.8"W), at Peñaflor, Seville province, Spain; collected by A. Archidona-Yuste, April 22, 2014; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR083-01).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers AR083-03-AR083-06); two females and one juvenile at Istituto per la Protezione Sostenibile delle Piante (IPSP),

Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (AR083-22); two females and two juveniles at Royal Belgian Institute of Natural Sciences, Brussels, Belgium(RIT 852); and two females and two juveniles at USDA Nematode Collection, Beltsville, MD, USA (T-6775p); collected by A. Archidona-Yuste, April 22, 2014.

Diagnosis

Xiphinema celtiense sp. nov. is a *Xiphinema* non-*americanum*-group species belonging to morphospecies Group 5 sensu Loof and Luc (1990). It is an apparently parthenogenetic species characterized by a moderate long body (4.7–5.5mm), assuming an open C-shaped when heat-relaxed; lip region hemispherical, both laterally and frontally rounded and separated from body contour by a slight depression, 13.5–16.0 µm wide; long odontostyle (145.0–167.0 µm); vulva situated at mid body; reproductive system didelphic-amphidelphic having both branches about equally developed, Z-differentiation containing almost 15 granular bodies, uterus tripartite with small crystalloid bodies in low number, and presence of prominent wrinkles in the uterine wall that may be confused with spiniform structures; female tail short, varying from hemispherical to convex-conoid shape, bearing two or three pairs of caudal pores; c´ ratio (0.8–1.0);males extremely rare, only one male was found, with moderately long spicules (74.0 µm) and 5 ventromedian supplements; and specific D2-D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KX244889-KX244890, KX244926-KX244927, and KX244943, respectively). According to the polytomous key of Loof and Luc (1990), the new species has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2-C7-D6-E6-F5-G34-H2-I3-J7-K2-I1.

Table 5.5 Identity matrix, percentage (%) of identical residues between (indels included) rDNA sequences amongst *Xiphinema* species. Above diagonal D2-D3 expansion segments of 28S rRNA and below diagonal internal transcribed spacer 1 (ITS1) region*.

	***Xiphinema* spp.**																		
***Xiphinema* spp.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1. *X. andalusiense* sp. nov.*		49	49	48	48	87	87	49	42	81	-	80	48	47	48	82	81	47	-
2. *X. celtiense* sp. nov.	86		85	80	79	49	50	87	58	50	-	47	81	78	72	50	48	70	-
3. *X. iznajarense* sp. nov.	87	94		79	79	48	49	86	57	49	-	48	81	77	72	49	48	70	-
4 *X. mengibarense* sp. nov.	86	93	93		76	49	49	82	59	50	-	48	76	75	73	49	48	70	-
5. *X. adenohystherum*	88	**95**	**96**	94		48	49	80	56	49	-	47	84	81	73	48	48	72	-
6. *X. baetica*	**98**	86	87	87	88		88	49	42	82	-	80	48	47	48	84	80	45	-
7. *X. cadavalense*	97	86	86	86	87	98		50	42	84	-	83	48	47	49	83	84	46	-
8. *X. cohni*	87	**96**	84	93	85	87	86		58	50	-	48	81	78	72	49	48	70	-
9. *X. conurum*	84	88	88	88	88	84	84	88		43	-	42	57	56	54	41	42	61	-
10. *X. coxi europaeum*	**96**	86	86	86	87	86	86	86	84		-	83	48	48	48	78	82	46	-
11. *X. gersoni*	87	**95**	**95**	94	**97**	87	86	**95**	88	86		-	-	-	-	-	-	-	-
12. *X. globosum*	**96**	86	88	87	88	86	86	86	85	**96**	87		46	46	47	77	84	46	-
13. *X. hispanum*	87	95	**96**	93	98	87	86	**95**	88	87	**96**	88		83	76	48	47	72	-
14. *X. hispidum*	88	97	**95**	94	95	88	87	**97**	89	87	**96**	88	**95**		74	48	46	73	-
15. *X. italiae*	87	93	93	94	94	87	87	93	88	87	**95**	87	**95**	94		47	47	70	-
16. *X. macrodora*	**97**	87	87	87	88	**97**	**97**	87	84	**96**	87	**96**	87	88	88		77	45	-
17. *X. pseudocoxi*	94	86	87	87	88	**95**	**95**	86	85	**96**	87	**98**	87	88	87	**96**		**45**	-
18. *X. pyrenaicum*	87	93	93	94	94	87	86	93	89	86	94	87	94	94	94	87	87		-
19. *X. sphaerocephalum*	87	94	85	94	94	87	87	94	89	87	94	87	**95**	**95**	94	88	87	94	-

* Similarity between sequences ≥ 95% are in bold letters.
(-) Sequences not available or comparison not carried out because of low homology between sequences.

Etymology

The species name is derived from originating Roman city of Peñaflor, *"Celti"*, where the type specimens were collected.

Description of taxa

Female:

Body cylindrical, with open C-shaped upon fixation. Cuticle 2.5–4.0 µm wide at mid-body, but thicker at tail tip, 6.5–11.0 µm wide. Lateral hypodermical chords visible throughout the length of the body, occupying about 23% of the corresponding maximum body diameter. Lip region hemispherical, both frontally and laterally rounded, slightly offset from body contour by a depression, 14.3 ± 0.8 (13.5–16.0) µm wide and 7.2 ± 1.4 (4.5–9.5) µm high. Amphidial fovea aperture extending for *ca* 58–78% of lip region diam. Guiding ring with average guiding sheath length of 15.5 µm. Odontostyle long, 1.4–1.8 times longer than odontophore, and the latter with well-developed flanges 13.0–16.5 µm wide. Pharynx very long occupying about 10–14% of body length, consisting of an anterior slender narrow part 379–510 µm long and extending to pharyngeal bulb, 126.0–168.0 µm long and 22.5–36.0 µm wide. Glandularium 110–155 µm long. Nucleus of dorsal pharyngeal gland (DN) located at beginning of basal bulb (11.5–16.1%), ventrosublateral nuclei (SVN) situated *ca* halfway along bulb (50.5–62.3%) (position of gland nuclei calculated as described by Loof and Coomans (1972). Vestigium small (tip of reserve odontostyle), 3 µm long, observed in all specimens studied in anterior region of slender part of pharynx. Cardia conoid, 8.5–17.5 µm long. Prerectum variable in length, 517–805 µm long, reaching about 10–16% of nematode body from the anus to anterior part. Rectum 36.5–44.0 µm long ending in anus as a small rounded slit. Reproductive system didelphic-amphidelphic with branches about equally developed. Each branch composed of an ovary 113–184 µm long, a reflexed oviduct with well-developed *pars dilatata oviductus* separated from uterus by a well-developed sphincter. Uterus tripartite composed of *pars dilatata uteri* followed by a tubular part containing in the proximal part a well-developed Z-differentiation with weakly muscularized wall, comprising 12–19 small granular bodies similar in size (Figures 5.6, 5.7F, G). Small crystalloid bodies similar in size and lower in number, mixed with abundant wrinkles from uterine wall, which may be confused as

spiniform structures, distributed over the entire length of the tube-like portion of uterus (Figures 5.6 and 5.7). In some specimens studied and in a proximal part of *pars dilatata uteri* spindle shaped sperm cells were observed. Ovejector well-developed 46.0–61.5 µm wide, and vagina perpendicular to body axis, 20.0–29.5 µm long or 27–42% of corresponding body diam. in lateral view. Vulva slit-like, situated slightly posterior the mid-body region. Tail short, always shorter than anal body diam., varying in shape from hemispherical to convex-conoid with rounded terminus, and bearing two or three caudal pores present on each side.

Male:

Extremely rare, only one male specimen was found in type locality. Male genital tract diorchic with testes containing multiple rows of different stages of spermatogonia. Tail short, convex-conoid with a broadly rounded terminus and thickened outer cuticular layer. Spicules moderately long and slightly curved ventrally; lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal and 4 mid-ventral supplements.

Description of juveniles:

All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Table 5.6, Figure 5.5) (Robbins *et al.* 1995, 1996). Specifically, J1 were characterised by position of replacement odontostyle just posterior to functional odontostyle, its tip touching or very close to base of functional odontostyle; tail conical elongate, ending in a knob-like expansion, more or less developed, separated from the anterior part of the tail by a depression more or less marked, but giving to the tail a very characteristic profile (Figures 5.6 and 5.7); c' ratio ≥ 4.0; and odontostyle length *ca* 75 µm. Tail morphology of second-stage juvenile similar to J1 expect to absence of knob-like expansion, and tail conoid and subdigitate with rounded terminus for third-stage juvenile. In J4 tail conoid with a short bulge rounded terminus (Figure 5.7). All juvenile developmental stages with tail becoming progressively shorter and stouter in each moult, and shorter distance from anterior end to guiding-ring in each moult (Table 5.6, Figure 5.7).

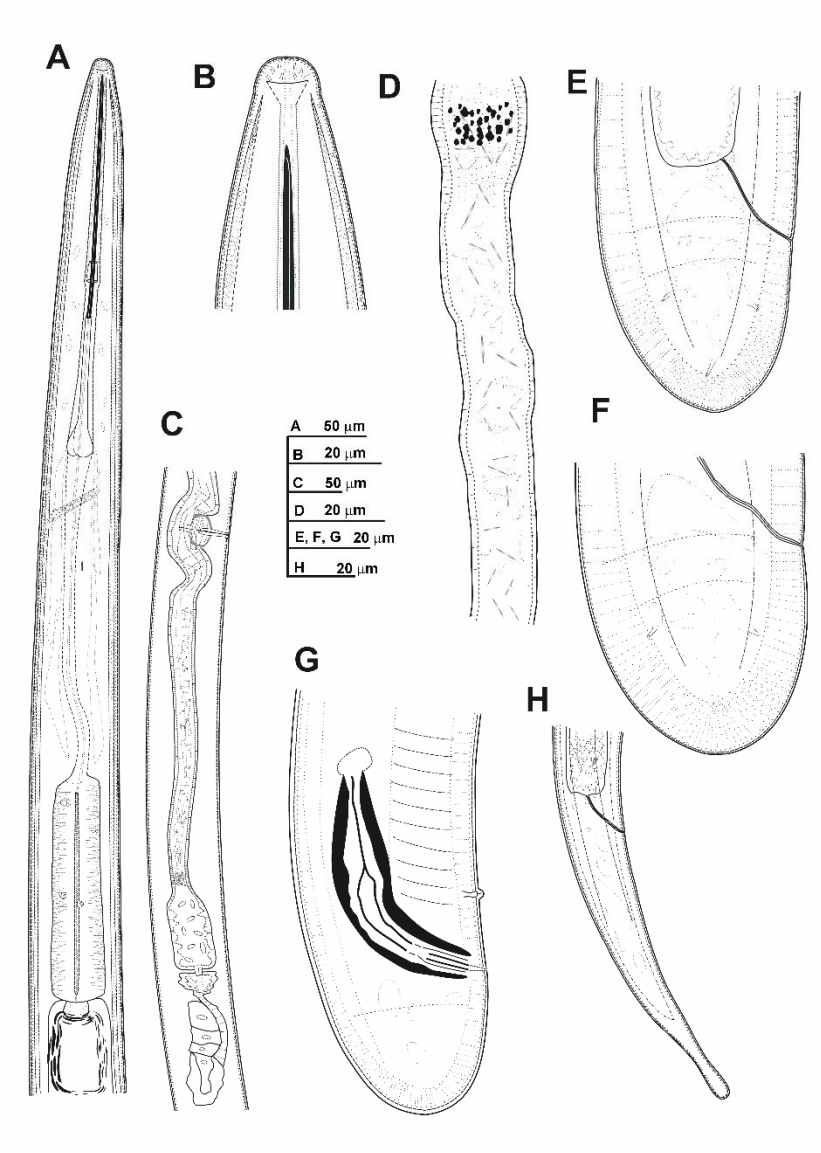

Figura 5.6: ine drawings of *Xiphinema celtiense* sp. nov., female paratypes, male and first-stage juvenile A) Pharyngeal region. B) Detail of lip region. C) Posterior female genital branch showing Z-differentiation. D) Detail of Z-differentiation. E-F) Female tails. G) Male tail. H) First-stage juvenile tail (J1).

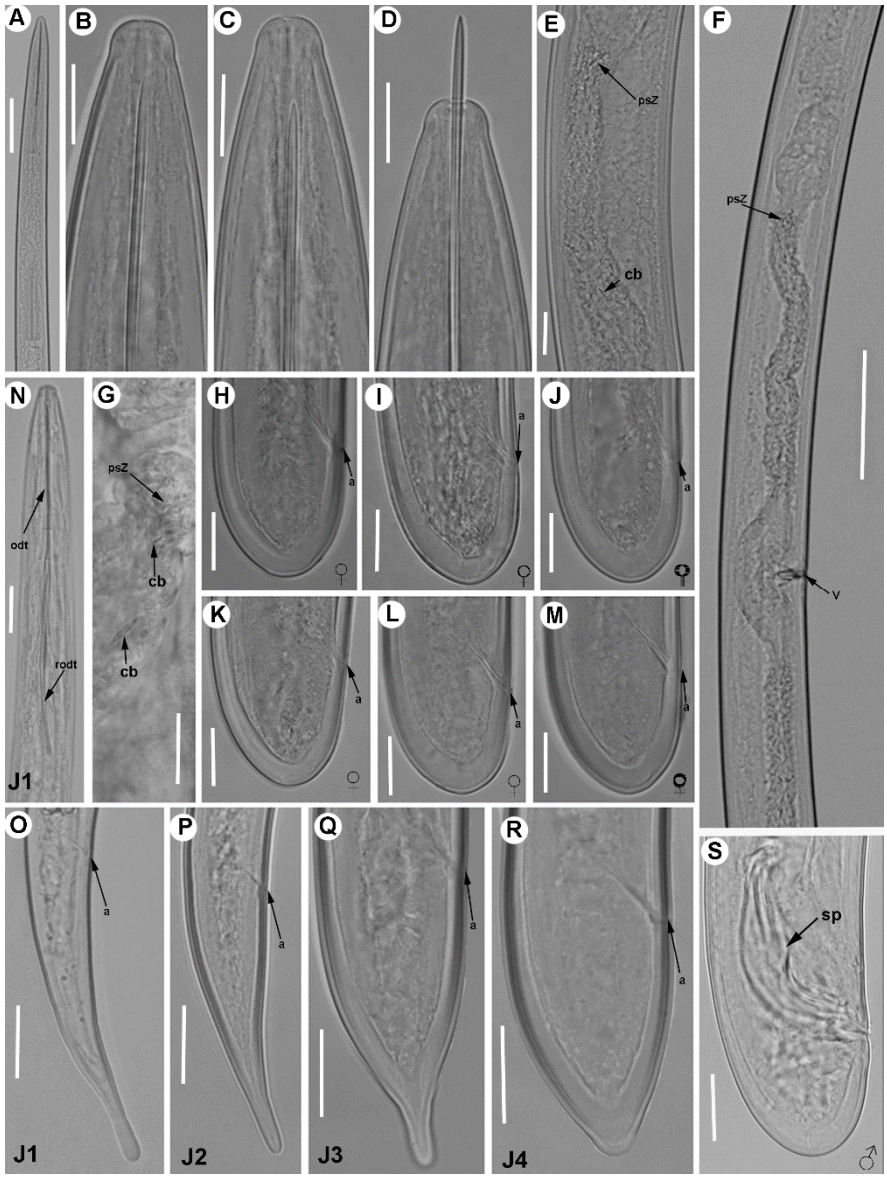

Figura 5.7: Light micrographs of *Xiphinema celtiense* sp. nov., female paratypes, male and juvenile stages A) Pharyngeal region. B±D) Female anterior regions. E) Detail of female genital track showing Z-differentiation. F) Detail of anterior female gonad showing Z-differentiation. G) Z-differentiation. H-M) Female tails. N) Detail of first-stage anterior region. O-R) First-, second-, third-, and fourth-stage juvenile (J1-J4) tails, respectively. S) Male tail with detail of spicules. Abbreviations: a = anus; cb = crystalloid bodies; gr = guiding-ring; odt = odontostyle; rodt = replacement odontostyle; sp = spicules; spZ = Z-differentiation; v = vulva. Scale bars = 20 µm.

Measurements, morphology and distribution:

Morphometric variability is described in Table 5.6 and morphological traits in Figures 5.5-5.7. In addition to the type locality, *Xiphinema celtiense* sp. nov. was found in the rhizosphere soil of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) in one additional locality belonging to Córdoba province (Table 5.1, Figure 5.1).

Relationships:

According to the polytomous key by Loof and Luc (1990) and sorting on matrix codes A (type of female genital apparatus), C (tail shape), D (c´ ratio), E (vulva position), F (body length), and G (total spear length (odontostyle + odontophore), *X. celtiense* sp. nov. groups with *X. iznajarense* sp. nov., *X. coronatum* Roca 1991, and *X. turcicum*. Firstly, *X. celtiense* sp. nov. can be clearly differentiated from these *Xiphinema* spp. in the absence of spiniform structures in the tubular part of uterus (Figures 5.8 and 5.9; Roca 1991, Gutiérrez-Gutiérrez *et al.* 2010). In addition, *X. celtiense* sp. nov. mainly differs from *X. iznajarense* sp. nov. by slightly lower a and c ratios (64.8–81.0, 97.5–143.9 vs 75.2–106.0, 119.4–175.5, respectively), posterior vulva position (50.0–55.0 vs 46.0–51.0%), a longer odontostyle and odontophore (145.0–169.0, 89.0–103.0 µm vs 132.0–151.0, 80.0–91.5 µm, respectively) resulting in a longer stylet length (241.0–263.05 vs 213.0–234.0 µm), a narrower lip region (13.5–16.0 vs 15.5–17.0 µm), frequency of males (extremely rare vs frequent), and the female and J1 tail shape (Figures 5.7-5.10, Tables 5.6 and 5.7). On the other hand, *X. celtiense* sp. nov. differs from *X. coronatum* in having a longer body length (4.7–5.5 vs 3.8–4.6mm), posterior vulva position (50.0–55.0 vs 47.1–51.8%), and presence vs absence of crystalloid bodies along uterus (Roca 1991). Finally, it can bemainly differentiated from *X. turcicum* by slightly higher a and c ratios (64.8–81.0, 97.5–143.9 vs 52.4–80.3, 83.1–128.0, respectively), presence vs absence of crystalloid bodies in the tubular portion of uterus, and different shape of J1 tail (dorsally convex and ventrally concave vs dorsally convex and ventrally almost straight) although in both species the tail ends in a knob-like expansion more or less separated from the anterior part of tail (Figures 5.6 and 5.7; Luc 1963, Gutiérrez-Gutiérrez *et al.* 2010).

In addition, *X. celtiense* sp. nov. is molecularly related to *X. hispanum* Lamberti, Castillo, Gómez Barcina and Agostinelli 1992 and *X. cohni*

Lamberti, Castillo, Gómez Barcina and Agostinelli 1992, but it can be clearly differentiated by a combination of characters discussed below. From *X. hispanum* it mainly differs in having a longer odontostyle (145.0–169.0 vs 131.2–142.3 µm), and female tail shape (hemispherical vs widely conical or dorso-ventrally convex) (Lamberti *et al.* 1992, Gutiérrez-Gutiérrez *et al.* 2010). And from *X. cohni* it mainly differs by the presence vs absence of Z-differentiation containing numerous granular bodies, and female tail shape (hemispherical vs convex-conoid or conical ending in a terminal bulge (Figures 5.6 and 5.7; Lamberti *et al.* 1992, Gutiérrez-Gutiérrez *et al.* 2013b).

Molecular divergence of the new species:

D2-D3 sequences from *X. celtiense* sp. nov. (KX244889-KX244890) differed with the closest related species, *X. hispanum* (GU725074) by 24 nucleotides and 3 gaps (97% similarity) and from *X. cohni* (KC567173, KX244901) from 27 nucleotides and 1 indel (97% similarity). Intraspecific variation of D2-D3 segments detected between the two studied population of *X. celtiense* sp. nov. consisted of 7 nucleotides (99% similarity), and no indels (Table 5.5). ITS1 (KX244926-KX244927) also showed some similarity, 87% (136 nucleotides and 28 indels) with *X. hispanum* (GU725061) and 86% (141 nucleotides and 34 indels) with *X. cohni* (KX244933). Intraspecific variation of the ITS1 for these sequences (KX244926-KX244927) was 44 nucleotides and 18 gaps, 95% similarity (Table 5.5). Some microsatellites were found in these sequences contributing to sequence variation. Finally, the partial 18S of *X. celtiense* sp. nov. (KX244943) showed a high level of similarity (99%) with several sequences deposited in GenBank such as *X. hispanum* (GU725083), *X. adenohystherum* (GY725084), and *X. nuragicum* (GU725080).

Table 5.6 Morphometrics of females, males and juvenile stages of *Xiphinema celtiense* sp. nov. from the rhizosphere of wild olive at several localities (Córdoba and Sevilla provinces) southern Spain[a].

Host/locality, sample code	Peñaflor (Sevilla, Spain) AR083							Adamuz (Córdoba, Spain) AR082
Characters/ratios[b]	Holotype	Paratype Females	Paratype Males	J1	J2	J3	J4	Female
n		20	1	6	6	6	6	3
L (mm)	5.0	5.0 ± 0.22	4.8	1.64 ± 0.11	1.92 ± 0.14	2.81 ± 0.14	3.76 ± 0.29	5.08 ± 0.32
		(4.7-5.5)		(1.46-1.80)	(1.75-2.11)	(2.61-3.00)	(3.36-4.11)	(4.7-5.4)
a	69.3	72.5 ± 3.9	78.3	50.9 ± 2.7	61.5 ± 4.4	63.3 ± 7.6	67.3 ± 5.9	69.2 ± 7.5
		(67.4-81.0)		(48.8-56.1)	(54.7-67.0)	(56.8-75.0)	(59.8-74.3)	(64.8-77.8)
b	8.1	8.1 ± 0.5	7.8	5.7 ± 1.2	4.8 ± 0.7	5.8 ± 0.3	6.5 ± 0.8	8.2 ± 0.3
		(7.0-9.4)		(4.2-7.3)	(3.8-5.8)	(5.4-6.2)	(5.8-7.8)	(8.0-8.5)
c	109.2	111.2 ± 11.8	132.0	18.4 ± 1.6	25.2 ± 2.9	39.2 ± 4.4	76.1 ± 5.2	109.4 ± 10.6
		(100.7-143.9)		(15.6-20.3)	(22.2-28.8)	(33.1-44.4)	(68.4-82.4)	(97.5-117.9)
c´	0.9	0.9 ± 0.1	0.8	4.2 ± 0.3	3.3 ± 0.4	2.1 ± 0.2	1.1 ± 0.1	0.9 ± 0.1
		(0.8-1.0)		(4.0-4.3)	(2.7-3.7)	(1.8-2.5)	(0.9-1.3)	(0.8-0.9)
V or T	50.5	51.1 ± 1.1	61.8	-	-	-	-	53.2 ± 1.6
		(50.0-53.5)		-	-	-	-	(52.0-55.0)
Odontostyle	148.0	158.4 ± 6.1	162.0	75.1 ± 2.4	90.8 ± 1.1	116.5 ± 5.0	137.9 ± 3.0	167.3 ± 2.9
		(145.0-167.0)		(72.0-76.0)	(89.0-92.0)	(108.0-121.5)	(133.5-141.0)	(164.0-169.0)
Odontophore	93.0	93.4 ± 3.2	99.5	51.3 ± 5.3	65.0 ± 1.7	76.0 ± 3.2	85.1 ± 3.6	92.5 ± 2.0
		(89.0-103.0)		(43.5-58.0)	(63.0-67.0)	(72.0-81.0)	(81.0-90.5)	(90.5-94.5)

Host/locality, sample code	Peñaflor (Sevilla, Spain) AR083							Adamuz (Córdoba, Spain) AR082
Characters/ratios[b]	Holotype	Paratype Females	Paratype Males	J1	J2	J3	J4	Female
Total stylet	241.0	251.8 ± 5.9	261.5	-	-	-	-	-
		(241.0-260.5)		-	-	-	-	
Replacement odontostyle	-	-	-	90.2 ± 2.0	115.5 ± 2.1	141.8 ± 5.0	166.7 ± 1.7	259.8 ± 4.7
				(89.0-91.0)	(112.0-118.0)	(136.0-150.0)	(165.0-169.5)	(254.5-263.5)
Lip region diam.	14.0	14.3 ± 0.8	14.5	9.4 ± 0.2	10.2 ± 0.4	11.5 ± 0.3	12.4 ± 0.7	15.3 ± 0.8
		(13.5-16.0)		(9.0-9.5)	(9.5-10.5)	(11.0-12.0)	(11.5-13.5)	(14.5-16.0)
Oral aperture-guiding ring	138.0	143.8 ± 6.1	142.0	57.5 ± 5.8	81.8 ± 5.3	101.8 ± 5.1	119.1 ± 10.7	149.0 ± 7.2
		(132.0-155.0)		(51.0-59.0)	(77.0-92.0)	(95.5-107.0)	(107.0-134.0)	(141.0-155.0)
Tail length	46.0	45.5 ± 3.6	36.5	89.1 ± 2.6	76.6 ± 3.4	72.7 ± 9.2	49.4 ± 3.3	46.5 ± 1.7
		(36.0-49.5)		(86.0-90.0)	(72.0-80.0)	(62.5-88.5)	(45.5-54.0)	(45.5-48.5)
J	10.5	9.6 ± 1.2	8.5	22.4 ± 4.7	24.4 ± 3.2	22.0 ± 2.3	8.0 ± 0.4	10.2 ± 2.1
		(7.0-12.0)		(14.5-26.5)	(22.0-30.0)	(20.0-25.5)	(7.5-8.5)	(8.5-12.5)
Spicules	-	-	74.0	-	-	-		
Lateral accessory piece	-	-	20.5	-	-	-		

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

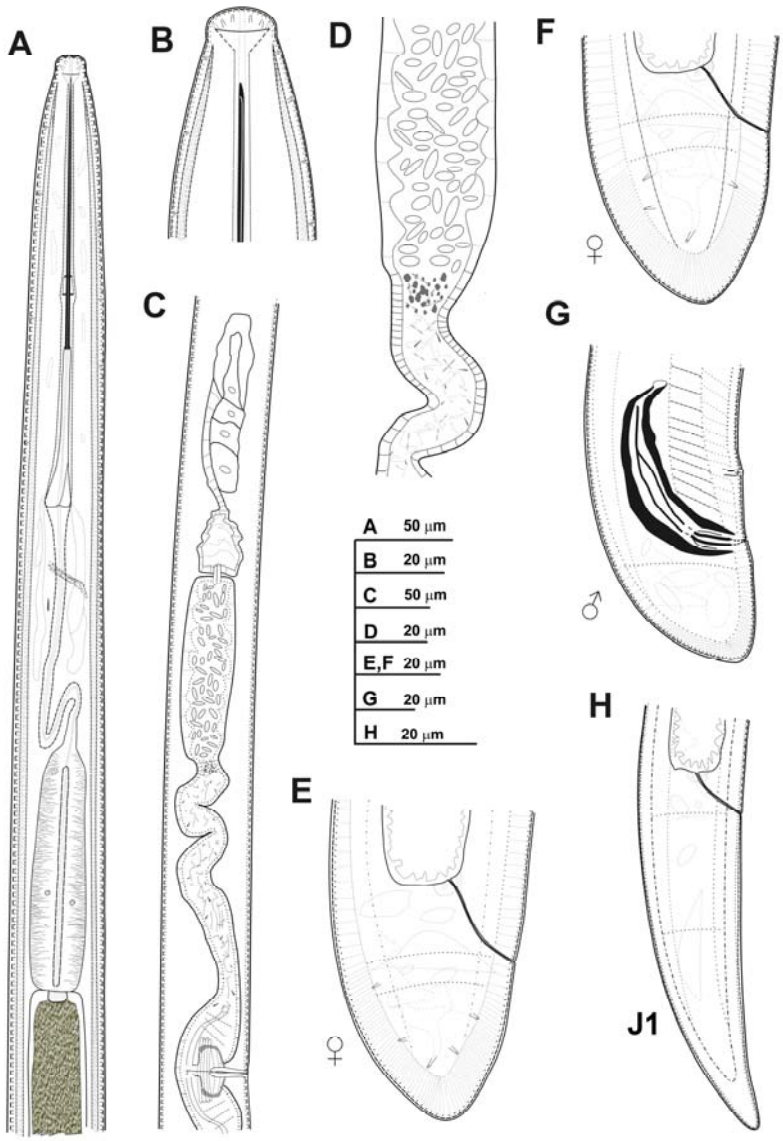

Figura 5.8: Line drawings of *Xiphinema iznajarense* sp. nov., female paratypes, male and first-stage juvenile A) Pharyngeal region. B) Detail of lip region. C) Anterior female genital branch showing Z-differentiation. D) Detail of Zdifferentiation. E-F) Female tails. G) Male tail. H) First-stage juvenile tail (J1).

3.2.3 *Xiphinema iznajarense* sp. nov.

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Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (37°15'39.4"N, 004°19'20.02"W), at Iznájar, Córdoba province, Spain; collected by J.E. Palomares-Rius, December 3, 2014; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number JAO-25-1).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers JAO-25-2-JAO-25-7); one female and one male at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (JAO-25-12); two females and one juvenile at Royal Belgian Institute of Natural Sciences, Brussels, Belgium(RIT 853); and two females, one male, and one juvenile at USDA Nematode Collection, Beltsville, MD, USA (T-6777p); collected by J.E. Palomares-Rius, December 3, 2014.

Diagnosis

Xiphinema iznajarense sp. nov. is an amphimictic species belonging to morphospecies Group 5 from *X.* non-*americanum*-group species sensu Loof and Luc (1990). It is characterized by a moderately long body (4.5–5.8mm), assuming an open C-shaped when heat-relaxed; lip region frontally rounded and almost laterally straight, usually low and distinctly set off from body contour, 15.5–17.0 µm wide; moderately long odontostyle

(132.0–151.0 µm); vulva position slightly anterior to mid body; reproductive system didelphic-amphidelphic with both branches about equally developed, Z-differentiation containing small and numerous granular bodies, uterus tripartite with small crystalloid bodies in higher number than small spiniform structures, and presence of prominent wrinkles from the uterine wall; female tail short and conoid, dorso-ventrally convex, ending in a rounded terminus and bearing four to five pairs of caudal pores; c´ ratio (0.7–1.1); males frequent with long spicules (*ca* 71 µm), and one pair of adanal supplement plus 4–5 pairs of ventromedian supplements; and specific D2-D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KX244891-KX244892, KX244928-KX244929, and KX244944, respectively). According to the polytomous key of Loof and Luc (1990), the new species has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2+3-C7-D6(5)-E5(6)-F5-G3-H2-I3-J6-K2-I2.

Etymology

The species refers to the type locality, Iznájar, where the species was detected.

Description of taxa

Female:

Habitus in specimens killed by gentle heat usually almost straight anterior to the vulva, more curved behind the vulva, occasionally open C-shaped. Cuticle 2.0–4.0 µm thick at mid-body, more thickened in the lip region (4.0–6.0 0 µm wide) and tail tip region (5.5–10.0 µm wide). Lateral hypodermical chords occupying about 26–46% of the corresponding maximum body diameter. Lip region hemispherical, broadly rounded frontally, usually low and offset from body contour by a shallow constriction; 15.5–17.0 µm wide and 5.5–7.5 µm high. Amphidial fovea aperture extending for *ca* 63–74% of lip region diam. and located at ca two-thirds of lip region height. Guiding ring with average guiding sheath length of 12.0 µm. Odontostyle moderately long, 1.5–1.8 times longer than odontophore, and the latter with well-developed flanges in the most of specimens studied, 11.5–22.0 µm wide. Pharynx consisting of an anterior slender narrow part 265–414 µm long, extending to a cylindrical, terminal pharyngeal bulb occupying ca 23–36% of total pharyngeal length, cylindrical, 117–153 µm long and 20–29 µm

wide. Glandularium 101–135 µm long. Nucleus of dorsal pharyngeal gland (DN) located at beginning of basal bulb (11.6–12.6%), ventrosublateral nuclei (SVN) situated *ca* halfway along bulb (50.5–57.8%) (position of gland nuclei calculated as described by Loof and Coomans (1972). In some specimens studied, vestigium (tip of reserve odontostyle), 2.5 µm long, observed in anterior region of slender part of pharynx. Cardia conoid and variable in length, 11.5–22.0 µm long. Prerectum reaching about 10–15% of nematode body from the anus to anterior part. Rectum29.5–38.0 µm long ending in anus as a small rounded slit. Reproductive system didelphic-amphidelphic with branches about equally developed. Each branch composed of a short ovary (63.5–122.0 µm long), a reflexed oviduct with well developed *pars dilatata oviductus* separated from uterus by a well-developed sphincter. Uteri tripartite, comprising a developed *pars dilatata uteri* continuing into a narrower, muscular tube-like portion, and a well-developed Z-differentiation with weakly muscularized wall and containing numerous small granular bodies. Uterine wall wrinkles present along uterus, being more numerous in the proximal part of *pars dilatata uteri* and ovejector (Figure 5.9E). Small spiniform structures and crystalloid bodies present, in low number, along uterus and observed when tubular part of uterus is wider and without wrinkles (Figures 5.8, 5.9G and 5.9H). In some specimens studied and in a proximal part of *pars dilatata uteri*, spindle-shaped sperm cells were observed, being variable in length (3.0–6.5 µm long). Ovejector well-developed 35.5–56.0 µm wide, vagina perpendicular to body axis, 18.0–24.0 µm long in lateral view. Vulva slit-like, pre-equatorial. Tail conoid and short, dorso-ventrally convex, ending in a rounded and broadly terminus, bearing in four to five pairs of caudal pores on each side.

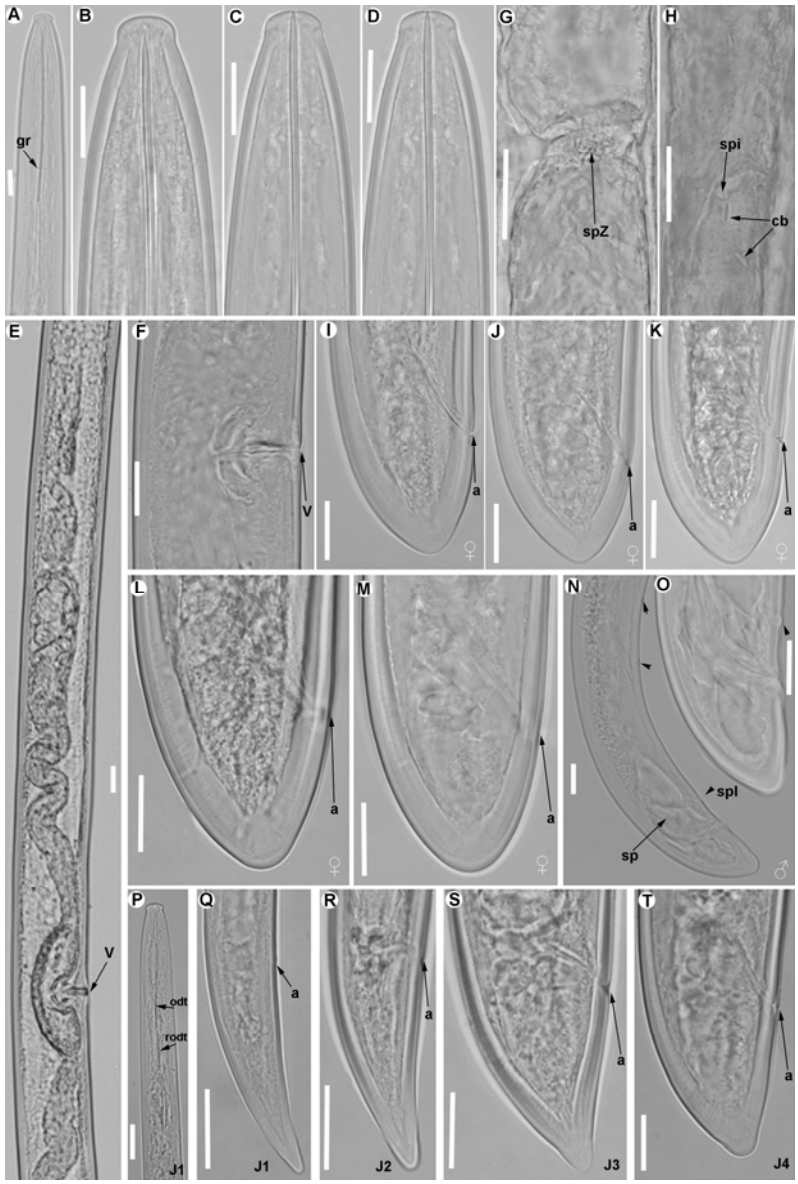

Figura 5.9: Light micrographs of *Xiphinema iznajarense* sp. nov. female paratypes, male and juvenile stages A-D) Female anterior regions. E) Detail of anterior female gonad. F) Vulval region. G-H) Detail of female genital track showing Z-differentiation. I-M) Female tails. N) Male tail with detail of spicules. P) Detail of first-stage anterior region. Q-T) First-, second-, third-, and fourth-stage juvenile (J1-J4) tails, respectively. Abbreviations: a = anus; cb = crystalloid bodies; gr = guiding-ring; odt = odontostyle; rodt = replacement odontostyle; sp = spicules; spi = spiniform structures; spl = ventromedian supplements; spZ = Z-differentiation; v = vulva. Scale bars = 20 µm.

Male:

Frequent but less abundant than female (ratio = 1: 2). Morphologically similar to female except for genital system and more curved posterior part of body. Male genital tract diorchic with testes containing multiple rows of different stages of spermatogonia. Tail short convex-conoid with short bulge rounded terminus and thickened outer cuticular layer (Figures 5.8, 5.9N and 5.9O). Spicules moderately long and slightly curved ventrally; lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal and 4–5 mid-ventral supplements.

Description of juveniles:

All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Table 5.7, Figure 5.5) (Robbins *et al.* 1995, 1996). In particular, J1 were characterised by position of replacement odontostyle just posterior to functional odontostyle, its tip touching or very close to base of functional odontostyle; tail blunty conoid elongate with a c´ ratio ≥ 3.8 (Figure 5.8 and 5.9Q); and odontostyle length *ca* 63 µm. Tail morphology in second-stage juvenile similar to J1 expect for the presence a slightly depression at the level of the hyaline region in both sides. On the other hand, the tail was conoid and subdigitate with a rounded terminus for J3, while for fourth-stage juvenile was conoid with rounded terminus and short bulge (Figure 5.9T). All juvenile developmental stages showed a tail becoming progressively shorter and stouter in each moult, and shorter distance from anterior end to guiding-ring in each moult (Table 5.7, Figures 5.9Q-T).

Measurements, morphology and distribution:

Morphometric variability is described in Table 5.7 and morphological traits in Figures 5.5, 5.8 and 5.9. *Xiphinema iznajarense* sp. nov. was only found in type locality, Iznájar (Córdoba province), being extracted from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (Table 5.1, Figure 5.1).

Table 5.7 Morphometrics of females, males and juvenile stages of *Xiphinema iznajarense* sp. nov. from the rhizosphere of cultivated olive at Iznájar (Córdoba province) southern Spain[a].

Host/locality, sample code	**cultivated olive, Iznájar (Córdoba province) JAO25**						
Characters/ratios[b]	**Holotype**	**Paratype Females**	**Paratype Males**	**J1**	**J2**	**J3**	**J4**
n		20	9	3	3	6	6
L (mm)	4.6	5.3 ± 0.37	5.4 ± 0.34	1.13 ± 0.06	1.74 ± 0.10	2.56 ± 0.18	3.87
		(4.5-5.8)	(4.8-5.8)	(1.07-1.17)	(1.61-1.86)	(2.25-2.77)	
a	85.4	89.7 ± 6.8	96.4 ± 9.3	49.3 ± 2.8	58.6 ± 10.3	62.3 ± 10.7	79.7
		(75.2-106.3)	(82.8-110.2)	(47.4-52.5)	(51.8-69.2)	(52.1-80.6)	
b	10.5	10.7 ± 0.8	10.0 ± 0.9	4.3 ± 0.5	5.8 ± 10.3	6.7 ± 0.9	8.8
		(9.7-12.8)	(8.4-11.3)	(3.8-4.9)	(5.3-6.5)	(5.3-8.1)	
c	121.8	134.9 ± 13.2	136.4 ± 10.3	21.5 ± 2.2	40.3 ± 13.0	56.1 ± 2.8	90.9
		(119.4-175.5)	(122.3-153.7)	(19.2-23.7)	(33.3-51.7)	(51.7-59.1)	
c´	0.9	1.0 ± 0.1	0.9 ± 0.01	2.9 ± 0.4	1.9 ± 0.4	1.4 ± 0.2	1.1
		(0.7-1.1)	(0.8-1.0)	(2.5-3.3)	(1.6-2.2)	(1.2-1.6)	
V or T	49.0	47.7 ± 1.3	58.5 ± 8.3	-	-	-	-
		(46.0-51.0)	(45.2-69.0)	-	-	-	-
Odontostyle	132.0	140.9 ± 4.7	140.0 ± 4.1	62.7 ± 6.8	81.0 ± 4.6	100.3 ± 3.0	126.0
		(132.0-151.0)	(132.0-145.5)	(55.0-68.0)	(78.5-85.0)	(96.0-103.5)	
Odontophore	81.0	84.6 ± 3.2	82.1 ± 4.6	49.2 ± 2.5	58.7 ± 1.8	66.3 ± 2.4	76.0
		(80.0-91.5)	(74.0-89.0)	(47.5-52.0)	(57.5-60.5)	(64.0-70.5)	
Total stylet	213.0	226.2 ± 5.2	222.1 ± 6.6	-	-	-	-
		(213.0-234.0)	(213.0-230.0)	-	-	-	-

Host/locality, sample code	cultivated olive, Iznájar (Córdoba province) JAO25						
Characters/ratios[b]	**Holotype**	**Paratype Females**	**Paratype Males**	**J1**	**J2**	**J3**	**J4**
Replacement odontostyle	-	-	-	80.8 ± 3.4	100.8 ± 0.4	120.8 ± 4.3	143.0
				(77.0-83.5)	(99.5-103.2)	(115.5-127.0)	
Lip region diam.	16.5	16.1 ± 0.4	15.7 ± 0.3	9.8 ± 0.3	11.2 ± 0.7	12.5 ± 0.0	14.0
		(15.5-17.0)	(15.5-16.0)	(9.5-10.0)	(10.5-11.5)	(12.5-12.5)	
Oral aperture-guiding ring	120.5	119.5 ± 3.6	121.6 ± 4.5	47.3 ± 2.0	65.0 ± 1.1	84.3 ± 2.3	103.0
		(113.0-125.0)	(117.0-129.0)	(45.5-49.5)	(63.5-66.5)	(81.0-88.0)	
Tail length	37.5	39.6 ± 2.8	39.4 ± 2.2	52.8 ± 3.1	50.8 ± 2.5	45.8 ± 2.4	42.5
		(32.5-44.0)	(35.5-42.0)	(49.5-55.5)	(48.5-52.0)	(43.0-48.5)	
J	11.5	10.1 ± 2.1	8.8 ± 1.1	11.2 ± 0.6	11.2 ± 3.2	10.9 ± 2.5	10.0
		(7.0-14.0)	(7.0-10.5)	(10.5-11.5)	(9.0-14.5)	(8.0-13.5)	
Spicules	-	-	70.7 ± 2.8	-	-	-	-
			(66.0-75.5)				
Lateral accessory piece	-	-	14.9 ± 0.9	-	-	-	-
			(13.5-16.0)				

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Relationships:

According to the polytomous key by Loof and Luc (1990) and sorting on matrix codes A (type of female genital apparatus), C (tail shape), D (c´ ratio), E (vulva position), F (body length), and G (total spear length (odontostyle + odontophore), *X. iznajarense* sp. nov. closely resembles with *X. celtiense* sp. nov., *X. coronatum* and *X. turcicum*. *Xiphinema iznajarense* sp. nov. can be differentiated from *X. celtiense* sp. nov. by the characters discussed above. From *X. coronatum* it differs in having a longer body (4.5–5.8 vs 3.8–4.6mm), higher a ratio (75.2–106.3 vs 65.5–75.5), a shorter odontophore and lower oral aperture-guiding ring distance (80.0–91.5, 113.0–125.0 µm vs 90.0–101.2, 142.3–154.1 µm, respectively), frequency of males (frequent vs extremely rare), presence vs absence of crystalloid bodies in the tubular portion of uterus, female tail shape (widely conical vs hemispherical), and shape of J1 tail (conoid elongate with rounded terminus vs a long clavate peg) (Figures 5.8 and 5.9; Roca 1991)). Finally, *X. iznajarense* sp. nov. can be differentiated from *X. turcicum* by slightly higher a and c ratios (75.2–106.3, 119.4–175.5 vs 52.4–80.3, 83.1–128.3, respectively), a shorter odontostyle length (132.0–151.0 vs 152.0–182.0 µm), the presence vs absence of crystalloid bodies along uterus, the frequency of males (frequent vs rare), the female tail shape (widely conical vs hemispherical), and shape of J1 tail (conoid elongate vs dorsally convex and ventrally almost straight ending in a knob-like expansion more or less separated from the anterior part of tail) (Figures 5.8 and 5.9; Luc 1963, Gutiérrez-Gutiérrez *et al.* 2010).

In addition, *X. iznajarense* sp. nov. is molecularly related to *X. hispidum* Roca and Bravo 1994 and *X. adenohystherum*, but it can be clearly differentiated by a combination of characters discussed below. From *X. hispidum* it can be differentiated by higher c ratio (119.4–175.5 vs 70.1–96.5), lower c´ ratio (0.7–1.1 vs 1.4–2.2), a longer odontostyle (132.0–151.0 vs 107.0–131.0 µm), and the presence vs absence of crystalloid bodies along uterus (Figures 5.8 and 5.9; Roca and Bravo 1994, Gutiérrez-Gutiérrez *et al.* 2011b). And from *X. adenohystherum* it clearly differs in having the presence vs absence of Z-differentiation containing numerous granular bodies, and presence vs absence of crystalloid bodies in the tubular portion of uterus (Figures 5.8 and 5.9; Lamberti *et al.* 1992, Gutiérrez-Gutiérrez *et al.* 2013b).

Molecular divergence of the new species:

D2-D3 region of *X. iznajarense* sp. nov. (KX244891-KX244892) was 97% similar (26 nucleotides and 1 indel) to *X. adenohystherum* (GU725075), *X. hispidum* (KC567181) and 95% similar (36 nucleotides and 2 indels) to *X. hispanum* (GU725074). No intraspecific variation of D2-D3 segments was detected amongst the studied individuals (100% similarity) (Table 5.5). Similarly, ITS1 (KX244928-KX244929) also showed some similarity with *X. hispanum* (GU725061), *X. adenohystherum* (GU725063) and *X. hispidum* (HM921367) with similarity values of 88% (131 nucleotides and 31 indels), 87% (145 nucleotides and 29 indels) and 84% (175 nucleotides and 52 indels), respectively (Table 5.5). ITS1 also showed a low intraspecific variation between the studied individuals, 9 nucleotides and no indels. The partial 18S of *X. iznajarense* sp. nov. (KX244944) closely matched with several species of *Xiphinema*, some of them were *X. adenohystherum* (GU725084), *X. hispanum* (GU725083), *X. gersoni* Roca and Bravo 1993 (Roca and Bravo 1993b) (KC567154) and *X. sphaerocephalum* Lamberti, Castillo, Gómez Barcina and Agostinelli 1992 (GU725082).

3.2.4 *Xiphinema mengibarense* sp. nov.

urn:lsid:zoobank.org:act:C42E7495-B8AD-42EF-BB3C-3F0E34476F2C

Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (38°01'21.72"N, 003°46'38.68"W), at Mengíbar, Jaén province, Spain; collected by J. Martín-Barbarroja, March 25, 2012; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number O3C4-01).

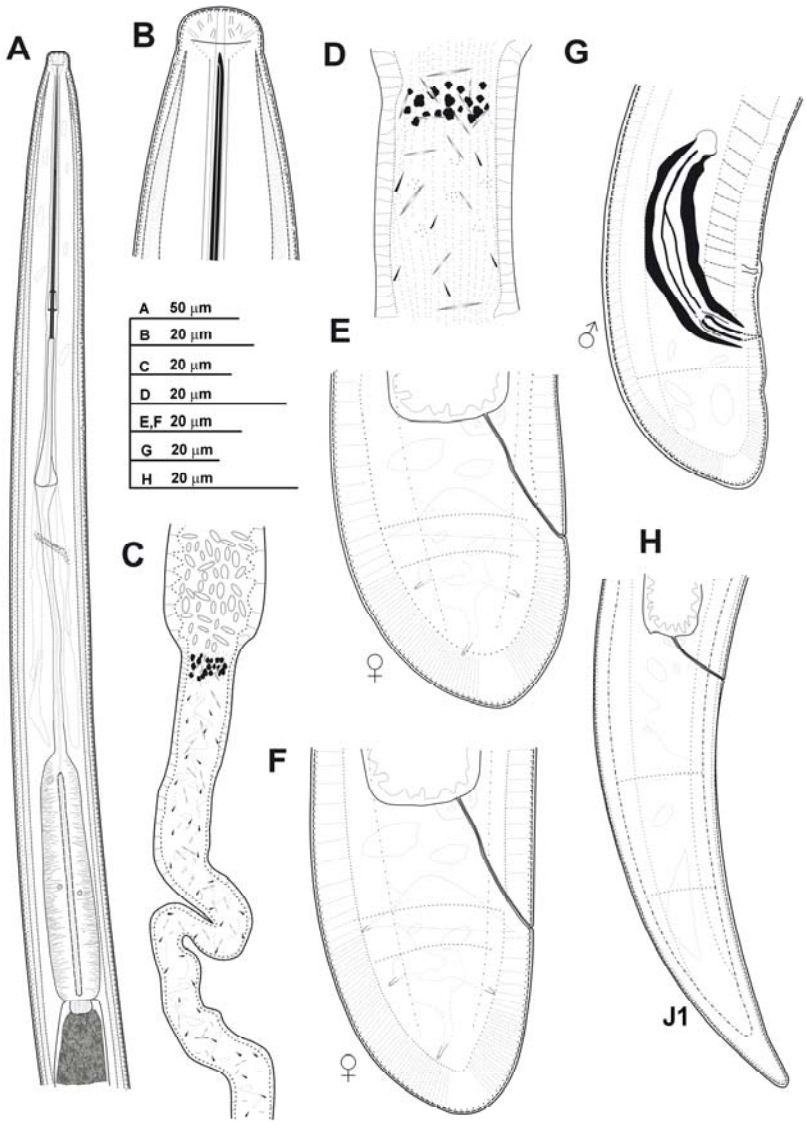

Figura 5.10: Line drawings of *Xiphinema mengibarense* sp. nov. female paratypes, male and first-stage juvenile. A) Pharyngeal region. B) Detail of lip region. C,D) Detail of Z-differentiation. E,F) Female tails. G) Male tail. H) First-stage juvenile tail (J1).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers O3C4-02-O3C4-08); one female, one male and one juvenile at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (O3C4-19); one female and one male at Royal Belgian Institute of Natural Sciences, Brussels, Belgium(RIT 854); and one female and one male at USDA Nematode Collection, Beltsville, MD, USA (T-6776p); collected by J. Martín-Barbarroja, March 25, 2012.

Diagnosis

Xiphinema mengibarense sp. nov. belongs to the *X.* non-*americanum* Group 5 in Loof and Luc (1990); and it is characterized by a moderate long body (3.8–4.8 mm), assuming an open C-shaped when heat-relaxed; lip region anteriorly rounded set off from body contour by a slightly depression, 12.5–15.5 µm wide and 5.5–8.5 µm high; guiding-ring located 104–122 µm from anterior end; moderately long odontostyle and odontophore (120.0–131.5, 73.0–83.5 µm, respectively); vulva slightly posterior to mid body; reproductive system didelphic-amphidelphic with both branches about equally developed including a *Z*-differentiation with muscularized wall and containing about 8–15 small granular bodies, uteri tripartite full of spindle shaped sperm in some specimens, and very small spiniform structures and crystalloid bodies in low number that in some specimens they can be confused with the wrinkles of the uterine wall; female tail broadly dorsally convex-conoid with rounded terminus, a short bulge, and a distinct terminal blind canal; c' ratio (0.7–1.1);males frequent but less abundant than females, with spicules 57.5–66.0 µm long and 5 to 6 ventromedian supplements; and specific D2-D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KX244893-KX244895, KX244930-KX244931, and KX244945, respectively). According to the polytomous key of Loof and Luc (1990), the species belongs to *Xiphinema* non-*americanum* Group 5 and has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2+3-C5a-D6(5)-E6(5)-F45-G32-H2-I2-J6-K2-I2.

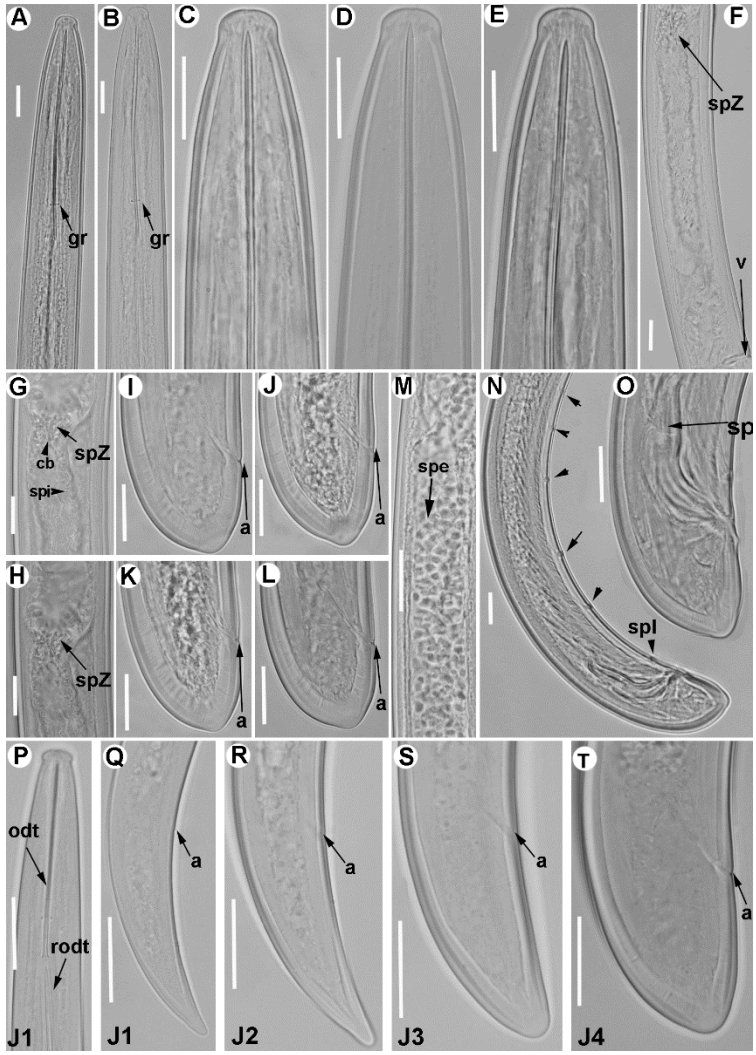

Figura 5.11: Light micrographs of *Xiphinema mengibarense* sp. nov. female paratypes, male and juvenile stages A-E) Female anterior regions. F-H) Detail of female genital track showing Z-differentiation. I-L) Female tails. M) Detail of male genital track showing sperm cells. N-O) Male tail with detail of spicules and ventromedian supplements. P) Detail of first-stage anterior region. Q-T) First-, second-, third-, and fourth-stage juvenile (J1-J4) tails, respectively. Abbreviations: a = anus; cb = crystalloid bodies; gr = guiding-ring; odt = odontostyle; rodt = replacement odontostyle; sp = spicules; spe = sperm cells; spi = spiniform structures; spl = ventromedian supplements; sss = spZ = Z-differentiation; v = vulva. Scale bars = 20 µm.

Table 5.8 Morphometrics of females, males and juvenile stages of *Xiphinema mengibarense* sp. nov. from the rhizosphere of cultivated olive at several localities (Jaén province) southern Spain[a].

Host/locality, sample code	**cultivated olive, Mengíbar (Jaén province) O3V4**						
Characters/ratios[b]	**Holotype**	**Paratype Females**	**Paratype Males**	**J1**	**J2**	**J3**	**J4**
n		20	11	5	6	6	6
L (mm)	4.6	4.3 ± 0.25	4.2 ± 0.28	1.24 ± 0.62	1.82 ± 0.96	2.40 ± 0.93	3.27 ± 0.44
		(3.8-4.8)	(3.6-4.6)	(1.18-1.34)	(1.71-1.89)	(2.32-2.57)	(3.21-3.34)
a	95.1	88.4 ± 5.2	94.0 ± 7.6	54.5 ± 1.5	66.8 ± 5.0	76.9 ± 9.1	80.7 ± 6.2
		(80.0-98.2)	(83.6-109.3)	(53.2-57.0)	(61.8-74.5)	(65.4-85.1)	(70.4-89.0)
b	9.2	8.5 ± 0.7	8.8 ± 0.8	5.7 ± 1.2	6.1 ± 0.7	6.6 ± 0.5	7.3 ± 0.3
		(6.5-9.8)	(7.3-10.2)	(4.8-7.7)	(5.4-7.3)	(6.2-7.6)	(6.9-7.7)
c	156.4	135.2 ± 13.7	121.0 ± 10.1	24.4 ± 2.1	38.7 ± 1.3	58.2 ± 2.0	91.0 ± 2.6
		(106.0-158.3)	(105.4-135.7)	(21.8-26.5)	(35.3-41.2)	(54.7-60.4)	(87.8-95.6)
c´	0.8	0.9 ± 0.1	1.0 ± 0.1	3.4 ± 0.4	2.3 ± 0.1	1.7 ± 0.1	1.1 ± 0.04
		(0.7-1.1)	(0.9-1.2)	(3.1-4.0)	(2.2-2.4)	(1.5-1.8)	(1.0-1.1)
V or T	54.0	52.1 ± 1.9	52.0 ± 3.1	-	-	-	-
		(48.5-57.0)	(45.7-57.2)	-	-	-	-
Odontostyle	126.5	125.0 ± 3.1	124.4 ± 4.3	52.8 ± 2.8	66.0 ± 1.4	84.9 ± 2.2	105.5 ± 2.8
		(120.0-131.5)	(117.0-131.5)	(48.5-56.0)	(63.5-69.0)	(83.0-88.5)	(101.5-110.0)
Odontophore	73.5	76.1 ± 2.5	72.0 ± 1.7	37.3 ± 3.6	46.4 ± 1.1	56.9 ± 1.7	65.5 ± 1.8
		(73.0-83.5)	(69.5-75.5)	(41.0-49.0)	(35.5-51.0)	(55.0-59.5)	(63.5-68.5)

Host/locality, sample code	cultivated olive, Mengíbar (Jaén province) O3V4						
Characters/ratios[b]	**Holotype**	**Paratype Females**	**Paratype Males**	**J1**	**J2**	**J3**	**J4**
Total stylet	200.0	201.1 ± 4.5	196.4 ± 4.3	-	-	-	-
		(194.5-215.0)	(188.0-203.0)	-	-	-	-
Replacement odontostyle	-	-	-	64.9 ± 0.9	87.0 ± 2.5	105.0 ± 3.9	127.8 ± 3.5
	-	-	-	(64.0-66.0)	(83.0-91.5)	(99.0-109.5)	(123.5-132.0)
Lip region diam.	14.5	13.9 ± 0.7	13.7 ± 0.5	8.7 ± 0.6	9.6 ± 0.4	10.6 ± 2.0	12.1 ± 0.2
		(12.5-15.5)	(12.5-14.0)	(8.0-9.5)	(8.5-10.0)	(10.0-11.5)	(12.0-12.5)
Oral aperture-guiding ring	114.0	112.1 ± 5.4	110.5 ± 4.3	44.2 ± 3.6	58.1 ± 0.4	75.3 ± 3.0	91.9 ± 4.2
		(104.0-122.0)	(104.0-118.5)	(41.0-49.0)	(57.0-61.0)	(71.0-79.0)	(87.5-99.5)
Tail length	29.5	32.1 ± 3.9	34.8 ± 1.8	50.9 ± 3.2	47.2 ± 3.9	41.3 ± 2.0	35.9 ± 1.0
		(27.0-42.0)	(32.5-38.5)	(47.5-55.5)	(42.5-53.5)	(39.5-44.5)	(34.0-36.5)
J	7.5	8.9 ± 1.2	7.9 ± 0.5	8.4 ± 1.2	8.7 ± 1.4	6.8 ± 0.8	7.9 ± 1.3
		(7.5-11.5)	(7.5-9.0)	(7.5-10.0)	(6.5-11.5)	(6.0-7.5)	(6.5-9.5)
Spicules	-	-	60.7 ± 2.6	-	-	-	-
			(57.5-66.0)	-	-	-	-
Lateral accessory piece	-	-	15.5 ± 1.6	-	-	-	-
	-	-	(13.5-18.0)	-	-	-	-

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Etymology

The species epithet refers to the type locality, Mengíbar, where the species was detected.

Description of taxa

Female:

Body cylindrical in an open C-shape when heat relaxed. Cuticle 3.1 ± 0.3 (2.0–4.5) µm thick at post-lip region, 2.8 ± 0.5 (2.0–4.0) µm wide at mid-body, but thicker just posterior to anus, 6.4 ± 1.8 (5.0–10.0) µm thick. Lateral chord 13.0 ± 4.8 (8.0–20.0) µm wide, occupying 17–42% of corresponding body diam. Lip region flatly rounded, slightly offset from body contour by a depression, 13.9 ± 0.7 (12.5–15.5) µm wide and 6.9 ± 0.8 (5.5–8.5) µm high. Amphidial fovea aperture extending for ca 64–78% of lip region diam. and located at *ca* two-thirds of lip region height. Guiding ring and guiding sheath variable in length depending on degree of protraction/retraction of stylet. Odontostyle moderately long, 1.5–1.7 times longer than odontophore, in the most specimens the latter with moderate-developed flanges, but in some specimens it was observed weaker, 8.5–14.0 µm wide. Pharynx composed by an anterior slender narrow flexible part 317–417 µm long, and a posterior muscular expanded part with three nuclei. Terminal pharyngeal bulb variable in length, 120–173 µm long and 19.5–29.5 µm wide. Glandularium 104–148 µm long. Nucleus of dorsal pharyngeal gland (DN) located at beginning of basal bulb (9.2–15.0%), ventrosublateral nuclei (SVN) situated *ca* halfway along bulb (45.7–58.0%) (position of gland nuclei calculated as described by Loof and Coomans (1972)). Cardia conoid, 10.4 ± 0.8 (8.0–12.5) µm long. Prerectum variable in length 586.2 ± 93.2 (444.0–772.0) µm long, or occupying 10–18% of body length. Rectum 18.5–36.0 µm long ending in anus as a small rounded slit. Reproductive system didelphic-amphidelphic with both branches about equally developed. Each branch composed of short reflexed ovary 65–97 µm long and a largely tubular oviduct with enlarged *pars dilatata oviduct* separated from uterus by a well-developed sphincter. Uteri tripartite, comprising a well-developed *pars dilatata uteri* continuing into a narrower, muscular tube-like portion including a Z-differentiation with weakly

muscularized wall and containing 8–15 small granular bodies. Wrinkles in uterine wall present, being more numerous in proximal part of *pars dilatata uteri*. Uteri and proximal part of *pars dilatata uteri* often with abundant spindle shaped sperm cells, 2.0–8.0 µm long. In some specimens, and when devoid of sperm, low numbers of small spiniform structures and crystalloid bodies seen along uterus, being more abundant about at Z-differentiation level. Ovejector well-developed, 36–47 µm wide, vagina perpendicular to body axis, 16.5–23.0 µm long or 34–47% of corresponding body diam. in lateral view. Vulva slit-like, situated in mid-body region. Tail broadly dorsally convex-conoid (slightly concave ventrally and hemispherical dorsally), with slightly bulging rounded terminus with a distinct terminal blind canal. Three to four caudal pores present on each side.

Male:

Functional, less abundant than females (ratio = 1: 2). Reproductive system diorchic with testes occupying 45–57% of body length, and spindle-shaped sperm. Spicules dorylaimoid, massive, well sclerotised, 57.5–66.0 µm long, ventrally curvedwith tubular lateral guiding pieces 13.5–18.0 µm long. One pair of adanal supplements located at 16.6 ± 1.2 (15.5–19.0) µm from cloacal opening and a series of four to five ventromedian supplements. Tail similar to that of female, dorsally more convex than female, and ending in a rounded terminus with short bulge.

Description of juveniles:

All four juvenile stages were found and detected using body length, length of replacement and functional odontostyle (Table 5.8, Figues 5.5 and 5.11) (Robbins *et al.* 1995, 1996). J1 were characterized by position of replacement odontostyle just posterior to functional odontostyle, its tip touching or very close to base of functional odontostyle; tail elongate, dorsally convex and ventrally concave with a slightly dorsal depression at hyaline region with a c' ratio ≥ 3.1 (Figures 5.10 and 5.11Q); odontostyle length *ca* 53 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages. Tail morphology in second and third juvenile stages similar to J1, becoming progressively shorter and stouter in each progressively moult. However, tail shape in fourth-stage similar into that of

female, broadly dorsally convex-conoid with slightly bulging rounded terminus (Figures 5.11Q and 5.11T).

Measurements, morphology and distribution:

Morphometric variability is described in Table 5.8 and morphological traits in Figures 5.5, 5.10 and 5.11. *Xiphinema mengibarense* sp. nov. was only found in type locality, Mengíbar (Jaén province), being extracted from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (Table 5.1, Figure 5.1).

Relationships:

According to the polytomous key by Loof and Luc (1990) and sorting on matrix codes A (type of female genital apparatus), C (tail shape), D (c´ ratio), E (vulva position), F (body length), and G (total spear length (odontostyle + odontophore), *X. mengibarense* sp. nov. groups with *X. herakliense* Tzortzakakis *et al*. 2015, *X. hispanum*, and *X. lanceolatum* Roca and Bravo 1993. Firstly, *Xiphinema mengibarense* sp. nov. can be differentiated from *X. herakliense* by higher a and c ratios (80.0–98.2, 106.0–158.3vs 59.0–75.0, 83.0–122.0, respectively), a shorter odontostyle (120.0–131.5 vs 127.0–157.0 µm), shorter spicules (57.5–66.0 vs 70.0–81.0 µm) (Tzortzakakis *et al.* 2015). On the other hand, *X. mengibarense* sp. nov. mainly differs from *X. hispanum* in having higher a ratio (80.0–98.2 vs 73.1–83.9), a shorter odontostyle (120.0–131.5 vs 131.2–142.3 µm), the number of spiniform structures present in the Z-differentiation (lower vs abundant), the presence vs absence of crystalloid bodies in the tubular portion of uterus, and the frequency of males (frequent *vs* rare) (Figures 5.10 and 5.11; Lamberti *et al.* 1992, Gutiérrez-Gutiérrez *et al.* 2010)). Finally, *X. mengibarense* sp. nov. can be differentiated from *X. lanceolatum* by higher a ratio (80.0–98.2 vs 50.5–75.5), a shorter odontostyle and odontophore (120.0–131.5, 73.0–83.5 µm vs 165.5–185.5, 90.0– 98.0 µm, respectively) resulting in a shorter stylet (194.5–215.0 vs 255.5–283.0 µm), a slightly narrower lip region (12.5–15.5 vs 14.5–18.0 µm), posterior vulva position (48.5–57.0 vs 43.5–50.0%), the presence vs absence of males, and the number of spiniform structures and crystalloid bodies (lower vs very abundant) (Figures 5.10 and 5.11; Roca and Bravo 1993a).

Molecular divergence of the new species:

D2-D3 region of *X. mengibarense* sp. nov. (KX244893-KX244895) was 94% similar to several *Xiphinema* species such as *X. italiae* (HM921351, 48 nucleotides and 12 indels), *X. pyrenaicum* Dalmasso, 1969 [82] (GU725073, 46 nucleotides and 15 indels) and *X. sphaerocephalum* (GU725076, 48 nucleotides and 10 indels). *Xiphinema mengibarense* sp. nov. showed a high homogeneity for the D2-D3 region (99% similarity, 2 nucleotides) in the three sampled populations (Table 5.5). The closest species in relation to ITS1 region were *X. hispanum* (GU725061) and *X. cohni* (KC567159), with a similarity of 84% (183 and 194 nucleotides and 55 and 65 indels, respectively) (Table 5.5). Low intraspecific variation for the ITS1 region (KX244930-KX244931) was detected among the studied population, 8 nucleotides and no indels. Finally, the partial 18S of *X. mengibarense* sp. nov. (KX244945) closely matched (99% similarity) those for *X. italiae* (FJ713154), *X. pyrenaicum* (GU725085) and *X. gersoni* (KC567154).

3.2.5 Morphology and morphometrics of species of known *Xiphinema* species

Morphological and morphometrical data, and molecular delineation (rDNA) of *X. adenohystherum*, *X. baetica*, *X. cohni*, *X. coxi europaeum*, *X. duriense*, *X. hispanum*, *X. hispidum*, *X. incertum*, *X. index* Thorne and Allen 1950, *X. italiae*, *X. lupini* Roca and Pereira 1993, *X. macrodora*, *X. madeirense* Brown, Faria, Lamberti, Halbrendt, Agostinelli and Jones 1993, *X. nuragicum*, *X. oleae*, *X. opisthohysterum* Siddiqi 1961, *X. pachtaicum*, *X. parapachydermum, X. plesiopachtaicum*, *X. rivesi* Dalmasso 1969, *X. santos* Lamberti, Lemos, Agostinelli and D'Addabbo 1993, *X. sphaerocephalum*, *X. turcicum*, *X. turdetanense*, and *X. vallense* have been previously recorded within studies of dagger and needle nematodes infesting olives and vineyards in southern Spain (Gutiérrez-Gutiérrez *et al.* 2013b, Archidona-Yuste *et al.* 2016a, b). Consequently, only D2-D3 sequences had been reported here for these samples. For other known species studied, representing the first molecular characterization and new records for olive or for Spain (*viz. X. cadavalense*, *X. conurum* and *X.*

pseudocoxi Sturhan 1984), a brief description and a morphometric comparison with previous records and paratypes is provided below (Figures 5.12-5.14, Table 5.9).

3.2.5.1 *Xiphinema cadavalense* Bravo and Roca 1995

The amphimictic population of *Xiphinema* collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) at Espiel (Córdoba province) corresponds fairly well with studied paratypes and original description of *X. cadavalense*. This population was characterised by a long body; lip region hemispherical, rounded both anteriorly and laterally and set off from body contour by slightly depression; long odontostyle and odontophore; reproductive system didelphic-amphidelphic with both branches about equally developed with a well-developed Z-differentiation with weakly muscularized wall and comprising 9–15 sclerotized bodies of variable size and petal shape, each one consisting of a large portion, irregularly spherical surrounded by a variable number of refractive pieces; spiniform structures and crystalloid bodies in very small size and low number present along the narrower and muscular tube-like of uterus; tail dorsally convex-conoid (dorsally convex and ventrally almost convex or slightly straight) ending in a terminal peg with blind canal (Figure 5.12, Table 5.9). The observations on the general morphology nematode indicate that this *Xiphinema* population belongs to the *X.* non-*americanum* Group 5 in Loof and Luc (1990), which agrees with the original description of *X. cadavalense* (Bravo and Roca 1995). In addition, female morphometrics fit with those provided in the original description, except in having slightly longer body and odontostyle length (5.2–5.9mm, 161.0–167.0 µm vs 4.0–5.3mm, 150.5–164.5 µm, respectively), posterior guiding ring position from oral aperture (149.5–167.0 vs 126.5–148.5 µm) (Bravo and Roca 1995). These differences may be due to geographical intraspecific variability. Up to our knowledge, this is the first report for Spain and confirms a wider distribution in the Iberian Peninsula, apart from original description in Portugal. According to the polytomous key of Loof and Luc (1990), this Spanish population of *X. cadavalense* has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2+3-C5a-D65-E56-F5-G4-H2-I3-JK-L1.

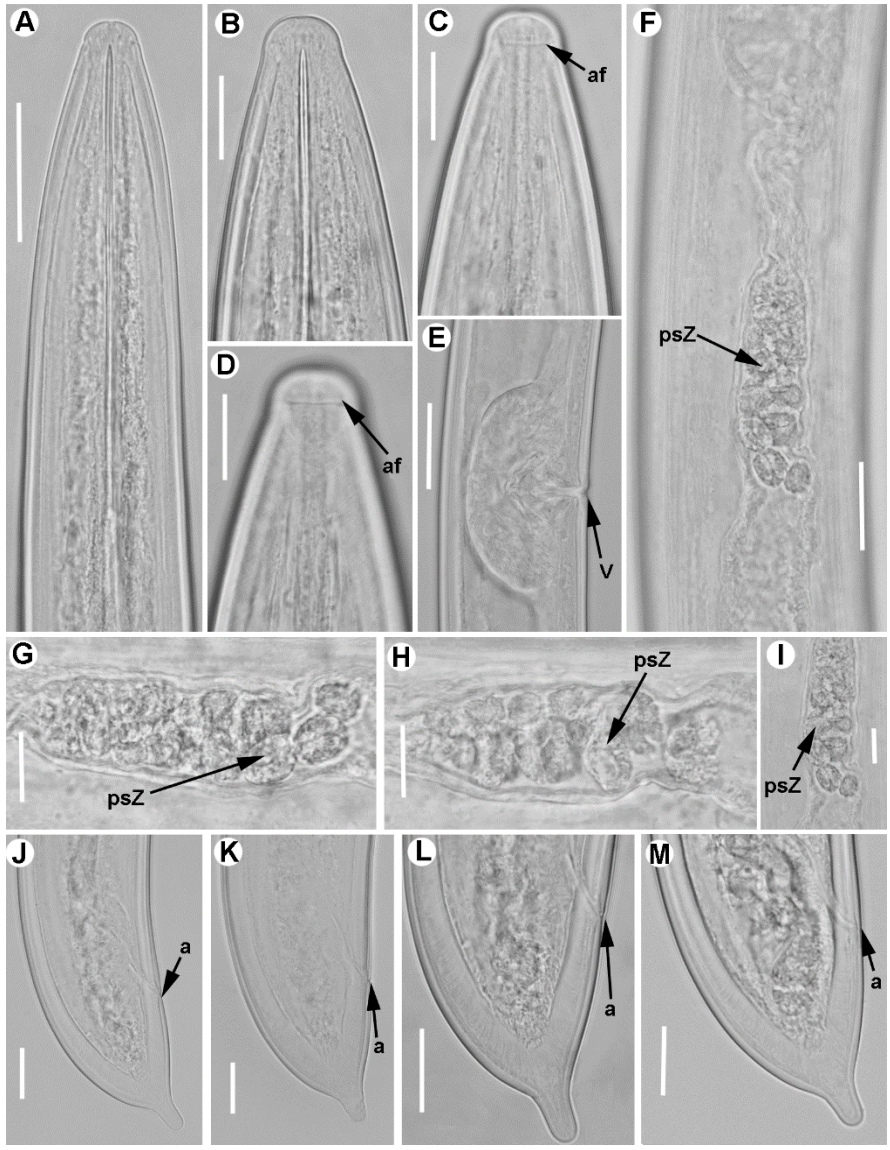

Figura 5.12: Light micrographs of *Xiphinema cadavalense* Bravo and Roca 1995 females from Spain A) Neck region. B-D) Female lip regions. E) Vulval region. F-I) Details of pseudo-Z organ. J-M) Female tails. Abbreviations: a = anus; af = amphidial fovea; psZ = pseudo-Z organ. Scale bars = 20 µm.

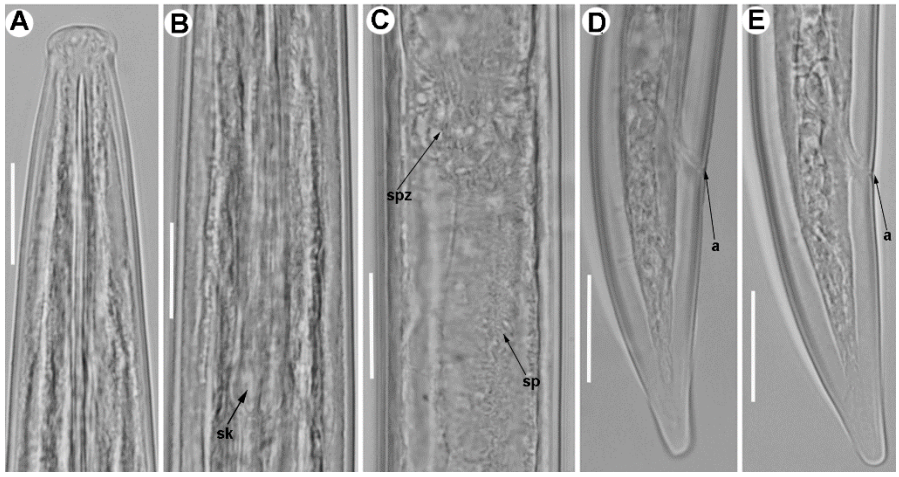

Figura 5.13: Light micrographs of *Xiphinema conurum* Siddiqi 1964 females from Spain A) Female lip region. B) Female anterior region showing detail of odontophore and flanges. C) Detail of female genital track showing Z-differentiation. D-E) Female tails. Abbreviations: a = anus; sk = flanges; sp = spiniform structures; spZ = Z-differentiation. Scale bars = 20 µm.

D2-D3 segments of *X. cadavalense* (KX244900) was 98% similar (14 nucleotides and no indels) to *X. baetica* (KC567168), 97% similar (24 nucleotides and 3 indels) to X. andalusiense sp. nov. (KX244884-KX244888) and 96% similar (30 nucleotides and 10 indels) to *X. macrodora* (KU171040, KU171242). ITS1 sequence (KX244932) region also agrees with results obtained from D2-D3, this sequence was 90% similar (105 nucleotides and 28 indels) to *X. baetica* (KC567157), 89% (121 nucleotides and 35 indels) to *X. andalusiense* sp. nov. (KX244921-KX244925) and 86% (157 nucleotides and 70 indels) to *X. macrodora* (KU171048). The partial 18S region of *X. cadavalense* (KX244946), was very similar to several sequences of *Xiphinema* spp., including *X. diversicaudatum* (Micoletzky, 1927) Thorne 1939 (JQ780346-JQ780349), *X. baetica* (KC567149) and *X. bakeri* Williams 1961 (AY283173).

3.2.5.2 *Xiphinema conurum* Siddiqi 1964

The Spanish population of this species from the rhizosphere of olive was characterised by a lip region rounded offset from the rest of the body by a conspicuous depression, two equally developed female genital branches, vulva slightly anterior to mid-body, uterus with uterine differentiation, presence of Z-differentiation with small granular bodies plus small spines (in low number), female tail conical, ventral profile nearly straight, dorsal profile regularly curved with rounded terminus (Figure 5.13). The morphology and morphometric of this population agree closely with the original description and redescription of the species by Siddiqi (1964) and Luc and Aubert (1985), likewise recently examined specimens from Soukra, Tunisia by Guesmi-Mzoughi *et al*. (2017). Up to our knowledge, this is the first report of this species for Spain.

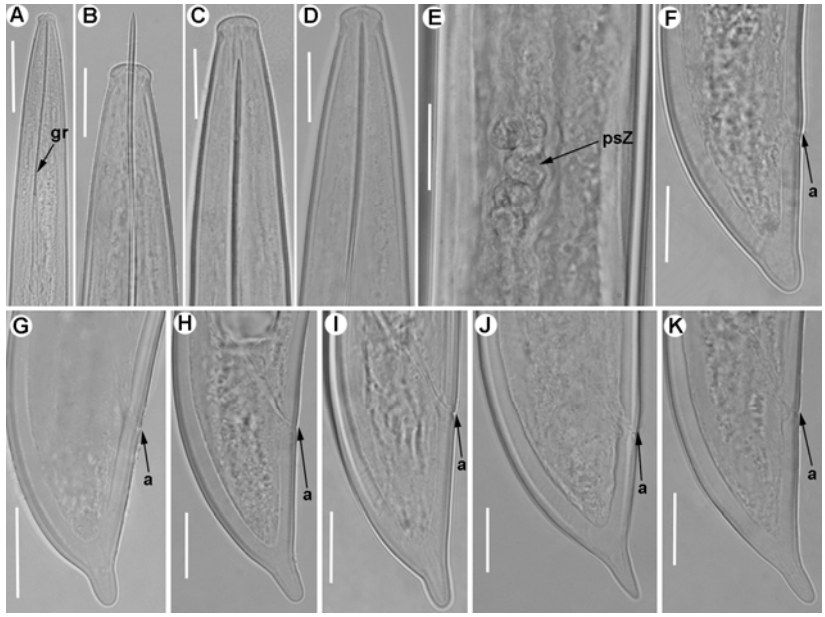

Figura 5.14: Light micrographs of *Xiphinema pseudocoxi* Sturhan 1984, females from Spain A) Neck region. B-D) Details of lip region. E) Detail of pseudo-Z organ. F-K) Female tails showing morphological variability. Abbreviations: a = anus; gr = guiding ring; psZ = pseudo-Z organ. Scale bars A = 50 µm; B-K = 20 µm.

Table 5.9 Morphometrics of females of *Xiphinema cadavalense* Bravo and Roca 1995, *Xiphinema conurum* Siddiqi 1964 and *Xiphinema pseudocoxi* Sturhan 1984 from the rhizosphere of cultivated and wild olives at several localities (Almería and Córdoba provinces) southern Spain[a].

Host/locality, sample code	*Xiphinema cadavalense* cultivated olive (Espiel, Córdoba) ST077	*Xiphinema conurum* cultivated olive (Uleila del Campo, Almería) ST045	*Xiphinema pseudocoxi* wild olive (Alcaracejos, Córdoba) AR095
Characters/ratios[b]	Females	Females	Females
n	6	2	10
L (mm)	5.5 ± 0.25	4.0 ± 0.30	4.1 ± 0.29
	(5.2-5.9)	(3.8-4.2)	(3.8-4.8)
a	65.2 ± 5.7	117.1 ± 8.7	80.6 ± 7.8
	(55.0-70.9)	(111.0-123.3)	(70.3-91.9)
b	8.8 ± 0.7	11.7 ± 0.6	9.5 ± 0.8
	(8.0-10.1)	(11.3-12.0)	(8.3-10.9)
c	97.9 ± 13.8	74.9 ± 7.1	85.5 ± 12.3
	(77.8-112.5)	(69.9-79.9)	(70.2-104.9)
c´	1.1 ± 0.1	2.4 ± 0.0	1.4 ± 0.2
	(0.9-1.2)	(2.4-2.5)	(1.2-1.7)
V	50.3 ± 1.5	46.8 ± 1.1	42.9 ± 1.2
	(48.5-52.5)	(46.0-47.5)	(41.0-45.0)
Odontostyle	163.9 ± 2.6	102.5 ± 2.1	120.1 ± 4.2
	(161.0-167.0)	(101.0-104.0)	(114.5-126.0)
Odontophore	105.3 ± 5.4	63.0 ± 0.7	69.4 ± 2.2
	(98.5-111.5)	(62.5-63.5)	(67.0-74.5)
Total stylet	269.2 ± 8.0	165.5 ± 2.8	189.5 ± 4.8
	(259.5-278.5)	(163.5-167.5)	(181.5-197.0)

Host/locality, sample code	*Xiphinema cadavalense* cultivated olive (Espiel, Córdoba) ST077	*Xiphinema conurum* cultivated olive (Uleila del Campo, Almería) ST045	*Xiphinema pseudocoxi* wild olive (Alcaracejos, Córdoba) AR095
Characters/ratios[b]	**Females**	**Females**	**Females**
Lip region diam.	17.8 ± 0.9	13.0 ± 0.7	12.2 ± 0.4
	(17.0-19.5)	(12.5-13.5)	(11.5-12.5)
Oral aperture-guiding ring	156.8 ± 6.7	87.8 ± 0.4	106.6 ± 5.2
	(149.5-167.0)	(87.5-88.0)	(98.0-113.5)
Tail length	56.9 ± 8.0	53.3 ± 1.1	48.5 ± 5.7
	(48.0-67.5)	(52.5-54.0)	(39.5-56.0)
J	22.5 ± 4.1	12.5 ± 1.4	17.5 ± 2.7
	(15.5-28.0)	(11.5-13.5)	(14.5-22.0)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; J (hyaline tail region length).

D2-D3 sequence for *X. conurum* (KX244902) matched well, 99% similar with former sequences from Tunisia deposited in GenBank (KX062671-KX062673); and ITS1 (KX244934) was 95–96% similar with former sequences from Tunisia deposited in GenBank (KX062696-KX062697). And partial 18S (KX244947) was provided for the first time in this research, being 99% similar to several Xiphinema spp. such as *X. nuragicum* (GU725081) or *X. israeliae* Luc, Brown and Cohn 1982 (Luc *et al.* 1982) (KJ802900), extending the molecular diversity of this species to newly studied area.

3.2.5.3 *Xiphinema pseudocoxi* Sturhan 1984

The amphimictic population of *Xiphinema* collected fromthe rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) at Alcaracejos (Córdoba province) agrees fairly well with original description of *X. pseudocoxi*. This population was characterised by a moderately long body in an open C-shaped after fixation; lip region distinct from the body contour by a depression, frontally rounded; female reproductive system didelphic-amphidelphic having both branches about equally developed; Z-differentiation with weakly muscularized wall formed by 6–10 globular bodies similar in size, and irregularly spherical surrounded by a variable number of refractive pieces; no spiniform structures and, crystalloid bodies nor sperm cells observed along uterus; female tail convex-conoid, varying slightly in shape, and ending in a terminal peg with a blind canal (Figure 5.14, Table 5.9). Based on the morphological character observations we confirm that this *Xiphinema* population belongs to the *X.* non-*americanum* Group 5 in Loof and Luc (1990), which is in agreement with the original description of *X. pseudocoxi* (Sturhan 1984). Additionally, female morphometrics fit with those provided in the original description and rather similar to data reported subsequently for other populations of Spain and Portugal, except for minor differences in nematode body and odontostyle length, which may be due to few specimens originally studied or geographical intraspecific variability (Sturhan 1984, Arias *et al.* 1987, Pereira and Roca 1992). This new Spanish population extends the species distribution in Europe, and confirms a wider distribution in the Iberian

Peninsula, apart from other populations from Spain, Portugal, and original description in Germany. According to the polytomous key of Loof and Luc (1990), the new Spanish population of *X. pseudocoxi* has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B2-C5a-D45-E4(5)-F4(5)-G2-H2-I3-J-K-L1.

Sequences for *X. pseudocoxi* (KX244915-KX244916) were obtained for the first time in this study. The closet species regarding D2-D3 segments of *X. pseudocoxi* (KX244915-KX244916) were *X. globosum* Sturhan 1978 (GU549474, 97% similar, 20 nucleotides and 3 indels), *X. diversicaudatum* (JQ780360-JQ780366, 96% similar) and *X. coxi europaeum* (KC567174-KC567176, 96% similar). Similarly, ITS1 region (KX244939-KX244940) also showed some similarity with *X. globosum* (GU549475, 88% similar, 127 nucleotides and 35 indels), *X. diversicaudatum* (HG969304, 87% similar, 154 nucleotides and 46 indels) and *X. coxi europaeum* (KC567160, 86% similar, 154 nucleotides and 43 indels). Finally, the partial 18S of *X. pseudocoxi* (KX244948) matched closely (99%) with several *Xiphinema* spp., such as *X. globosum* (GU549476), *X. diversicaudatum* (EF538761), *X. bakeri* (AY283173), *X. vuittenezi* Luc, Lima,Weischer and Flegg 1964 (EF614267) and *X. index* (AY687997).

3.3 Phylogenetic relationships of the *Xiphinema* spp.

The amplification of D2-D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial 18S rRNA yielded a single fragment of approximately 800 bp, 1000 bp, and 1800 bp, respectively, based on gel electrophoresis. Sequences from other species of *Xiphinema* spp. obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were used for further phylogenetic studies. Sequences for *X. andalusiense* sp. nov., *X. cadavalense*, *X. celtiense* sp. nov., *X. duriense*, *X. iznajarense* sp. nov., *X. mengibarense* sp. nov., *X. opisthohysterum* and *X. pseudocoxi* were obtained for these species in this study. On the other hand, sequences from *X. adenohystherum*, *X. cohni*, *X. conurum*, *X. hispanum*, *X. hispidum*, *X. incertum*, *X. index*, *X. italiae*, *X. nuragicum*, *X. parapachydermum*, *X. turcicum* and *X. turdetanense* matched well with former sequences deposited in GenBank, and spread out the molecular diversity of these species to the newly studied areas.

Phylogenetic relationships among *Xiphinema* non-*americanum*-group species inferred from analyses of D2-D3 expansion segments of 28S, ITS1, and the partial 18S rDNA gene sequences using BI are given in Figures 5.15-5.17, respectively. Poorly supported clusters were not explicitly labelled. The 50% majority rule consensus 28S rRNA gene BI tree of *X.* non *americanum*-group spp. based in a multiple edited alignment including 103 sequences and 753 total characters showed two clearly separated (PP = 1.00) major clades (Figure 5.15). Clade I was not well supported. This clade grouped thirty-five species including morphospecies from Groups 1, 4, 5, 6, 7 and 8. This major clade grouped three of the four new species described in this study: *X. celtiense* sp. nov. from wild olive, and *X. iznajarense* sp. nov. and *X. mengibarense* sp. nov. from cultivated olive. *Xiphinema celtiense* sp. nov. formed a well-supported subclade (PP = 1.00) with *X. cohni* (KC567173, (KX244901) and *X. hispanum* (GU725074, KX244905), this clade was related (PP = 1.00) with another subclade which was formed by *X. iznajarense* sp. nov. (KX244891-KX244892), *X. adenohystherum* (KX244896-KX244898, GU725075), *X. hispidum* (KC567181, KX244906) and *X. gersoni* (KC567180). Finally, *X. mengibarense* sp. nov. formed a low-supported subclade (PP = 0.76) with *X. italiae* (AY601613, KX244911-KX244912), *X. pyrenaicum* (GU725073), and *X. meridianum* Heyns 1971 (KX062678-KX062679). Clade II was moderately supported (PP = 0.86) and was formed by twenty species, all of them from the morphospecies Group 5, except *X. bakeri* and *X. index* which belong to Groups 7 and 8, respectively. This clade grouped sequences from the new species *X. andalusiense* sp. nov. (KX244884-KX244888) and the new accessions from *X. cadavalense* (KX244900), *X. conurum* (KX244902), and *X. pseudocoxi* (KX244915-KX244916). *Xiphinema andalusiense* sp. nov. (KX244884-KX244888) from wild olive occupied a superior position within the clade II forming a well-supported subclade (PP = 1.00) with *X. cadavalense* (KX244900) from cultivated olive, *X. baetica* (KC567167, KX244899) and *X. macrodora* (KU171040, KU171042). Finally, *X. pseudocoxi* (KX244915-KX244916) was phylogenetically related to *X. globosum* (GU549474) forming a well-supported clade (PP = 0.99).

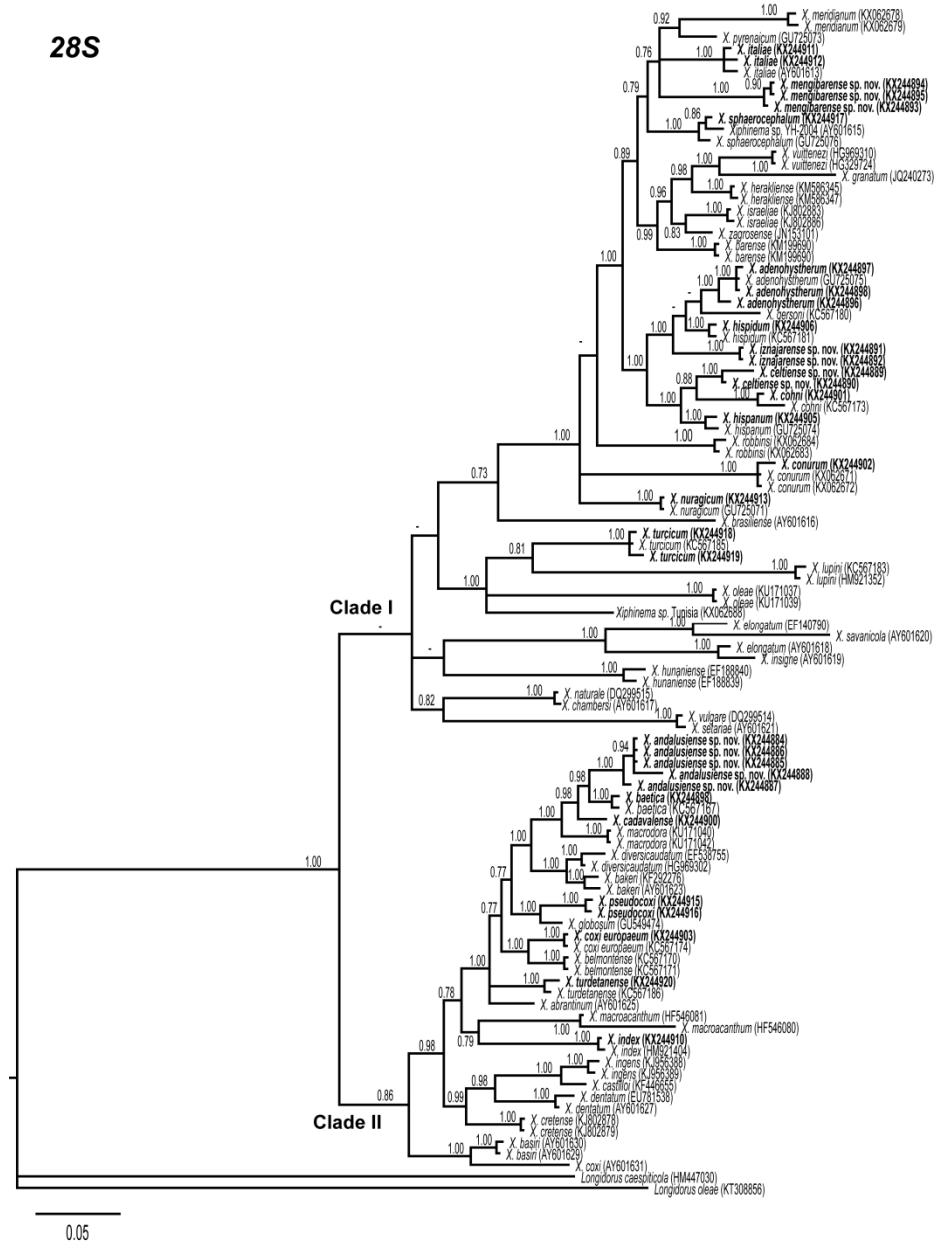

Figura 5.15: The 50% majority rule consensus tree from Bayesian inference analysis generated from the D2-D3 of 28S rRNA gene dataset of *Xiphinema* spp. with the GTR+I+G model. Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site.

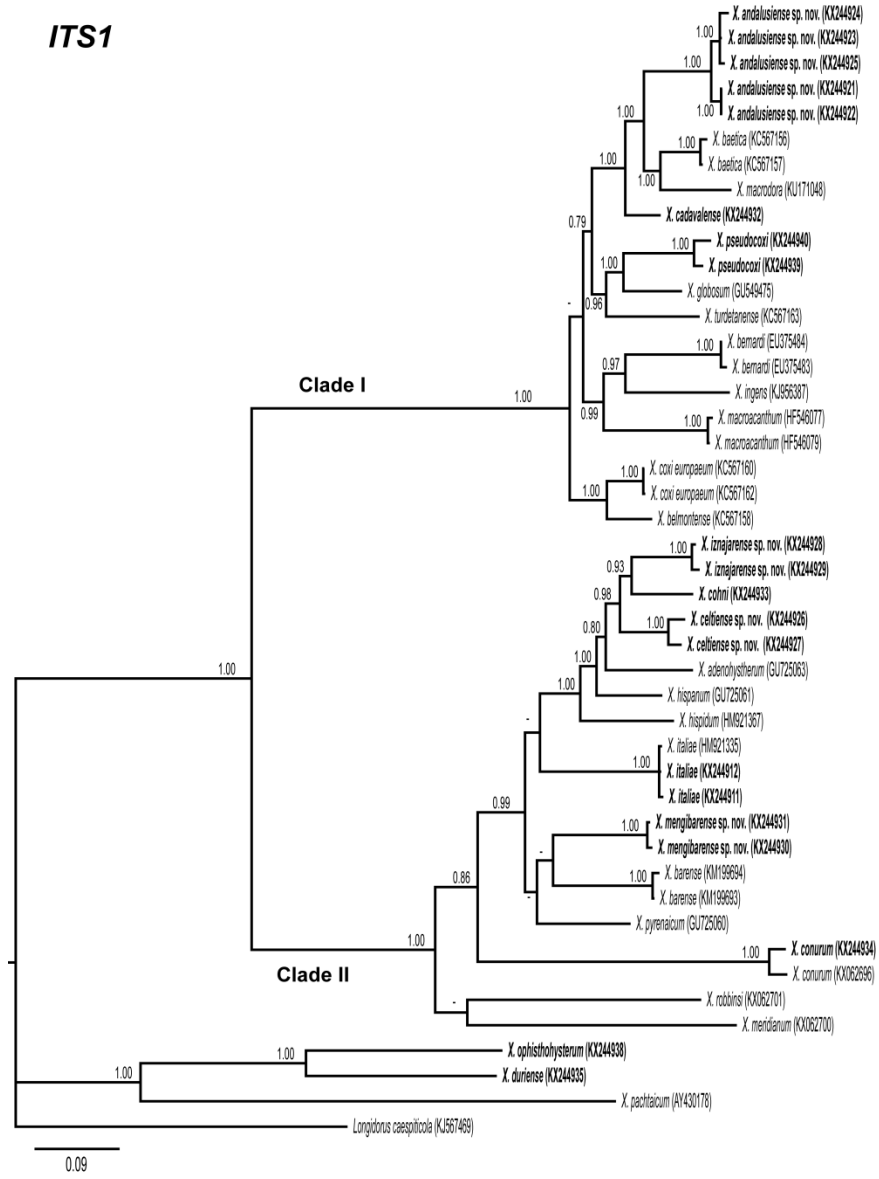

Figura 5.16: The 50% majority rule consensus trees from Bayesian inference analysis generated from the ITS rRNA gene dataset of *Xiphinema* spp. with the GTR+I+G model. Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site.

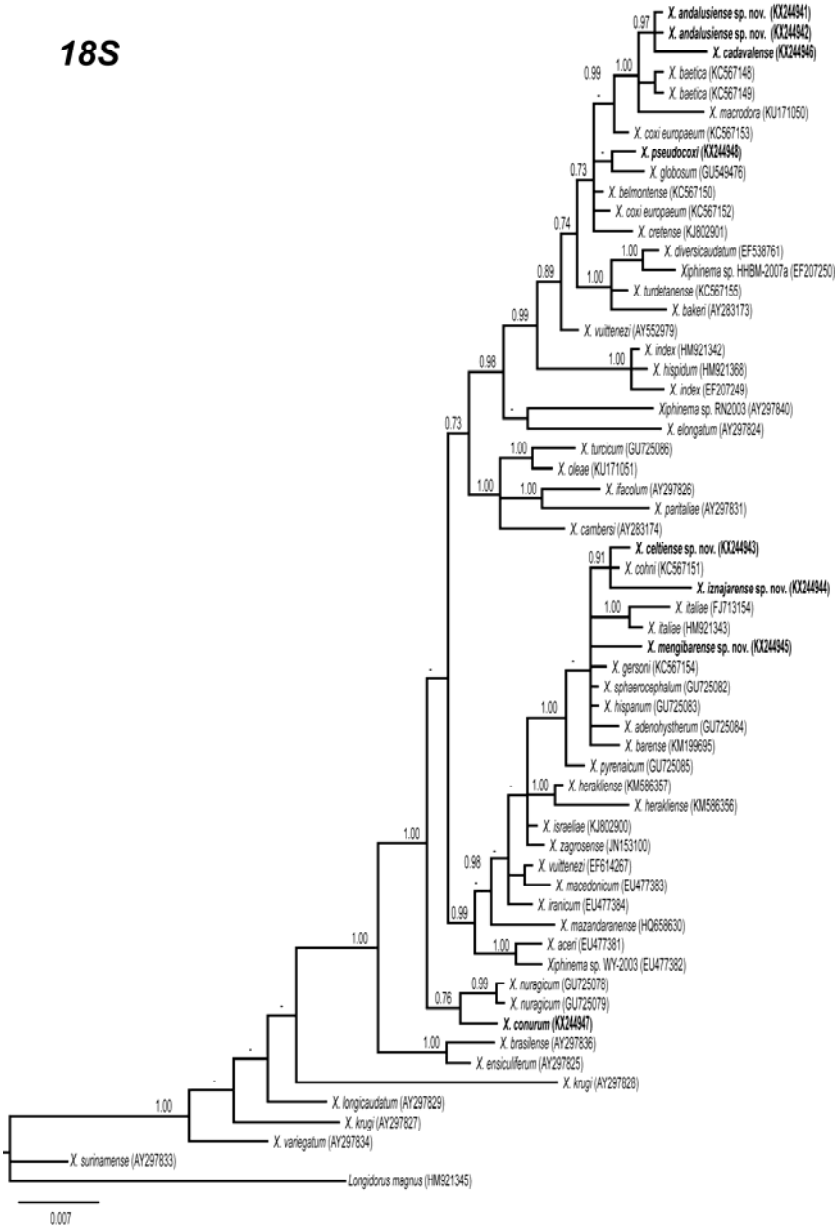

Figura 5.17: The 50% majority rule consensus trees from Bayesian inference analysis generated from the partial 18S rRNA gene dataset of *Xiphinema* spp. with the TIM3+I+G model. Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site.

Difficulties were experienced with alignment of the ITS1 sequences due to scarce similarity, thus, only related sequences were used. The alignment generated for the 45 sequences of ITS1, comprising several *X.* non-*americanum*-group species, was 1113 characters after discarding ambiguously aligned regions from the alignment. Two new accessions were used as outgroup, *X. duriense* (KX244935) and *X. opisthohysterum* (KX244938). The 50% majority rule consensus BI tree of *X.* non-*americanum*-group spp. showed two major clades (PP = 1.00) similar to those obtained for D2-D3 region (Figure 5.16). Clade I was formed by twelve *Xiphinema* species from morphospecies Group 5 including *X. andalusiense* sp. nov. (KX244921-KX244925), *X. pseudocoxi* (KX244939-KX244940) and *X. cadavalense* (KX244932). *Xiphinema andalusiense* sp. nov. (KX244921-KX244925) and *X. cadavalense* (KX244932) clustered with *X. baetica* (KC567156-KC567157) and *X. macrodora* (KU171048) in a well-supported subclade (PP = 1.00), these results agree with the results obtained with D2-D3 region. *Xiphinema pseudocoxi* and *X. globosum* were also phylogenetically related to this marker and they were placed in a well-supported subclade (PP = 1.00) which was related (PP = 0.96) at the same time with *X. turdetanense* (KC567163). Clade II grouped thirteen species from different morphospecies Groups 1, 4, 5 and 7, including *X. celtiense* sp. nov., *X. iznajarense* sp. nov. and *X. mengibarense* sp. nov. *Xiphinema iznajarense* sp. nov. (KX244928-KX244929), and *X. celtiense* sp. nov. (KX244926-KX244927) clustered together with *X. cohni* (KX244933), *X. adenohystherum* (GU725063), *X. hispanum* (GU725061) and *X. hispidum* (HM921367) as occurred in the D2-D3 tree. Finally, *X. mengibarense* sp. nov. (KX244930-KX244931) formed a low-supported subclade with *X. barense* Lamberti, Roca, Agostinelli and Bleve-Zacheo 1986 (KM199694-KM199693) and this subclade was related to *X. pyrenaicum* (GU725060) although this relation also was poorly supported. The new accessions for *X. duriense* (KX244935) and *X. opisthohysterum* (KX244938) clustered together with *X. pachtaicum* (AY430178) as an outgroup, all of Them from the *X. americanum*-group (Figure 5.17).

The 50% majority rule BI tree of a multiple alignment including 60 18S sequences and 1647 bp long showed several major clades (Figure 5.17). Additionally, in the D2-D3 and ITS1 trees, *X. andalusiense* sp. nov. (KX244941-KX244942) clustered with *X. cadavalense*, *X. macrodora* and

X. baetica within the same well-supported subclade (PP = 1.00). Phylogenetic inferences based on 18S also suggest that *X. pseudocoxi* and *X. globosum* are related species, although this relation was poorly supported (Figure 5.17). Finally, X. iznajarense sp. nov. (KX244944), *X. celtiense* sp. nov. (KX244943) and *X. mengibarense* sp. nov. (KX244945) clustered in this case with *X. cohni* (KC567151), *X. hispanum* (GU725083), *X. adenohystherum* (GU725084), *X. italiae* (FJ713154, HM921343), *X. barense* (KM199695), *X. gersoni* (KC567154), *X. sphaerocephalum* (GU725082), and *X. pyrenaicum* (GU725085) within a well-supported subclade (PP = 1.00).

4. Discussion

This study aimed to get knowledge and a better understanding on the occurrence, abundance and biodiversity of dagger nematodes of the genus *Xiphinema* associated with wild and cultivated olives in southern Spain, as well as their distribution and molecular phylogeny. This was conducted in an extensive and systematic nematological survey that included 211 locations and 453 sampling sites. We found 385 Spanish populations of *Xiphinema* spp. infesting olive soils. We described four new *Xiphinema* species, enlarging the diversity of *Xiphinema* species in the Iberian Peninsula which is in agreement with previous data obtained for the phylogeny and biogeography of the genus *Xiphinema* and *Longidorus* in the Euro-Mediterranean region (Navas *et al.* 1990, 1993, Gutiérrez-Gutiérrez *et al.* 2011b, 2013b, Archidona-Yuste *et al.* 2016c, b, a). To the date, to our knowledge, this work is the largest phylogenetic analysis of the genus *Xiphinema* based on nuclear rDNA markers.

The genus *Xiphinema* is one of the most diverse PPN associated with olive, with twenty species (*viz. X. aequum* Roca and Lamberti 1988, *X. barense*, *X. californicum* Lamberti and Bleve-Zacheo 1979, *X. cretense* Tzortzakakis *et al*. 2014, *X. diversicaudatum*, *X. duriense*, *X. elongatum* Schuurmans-Stekhoven and Teunissen (1938), *X. herakliense*, *X. incertum, X. index*, *X. ingens* Luc 1963, *X. italiae*, *X. israeliae*, *X. lusitanicum* Sturhan 1983, *X. macroacanthum* Lamberti, Roca and Agostinelli 1990, *X.*

macrodora, *X. madeirense*, *X. nuragicum*, *X. oleae*, *X. opisthohysterum*, *X. pachtaicum*, *X. parapachydermum*, *X. plesiopachtaicum*, *X. rivesi*, *X. sahelense* Dalmasso 1969, *X. turcicum*, *X. vallense*, *X. vuittenezi* and several unidentified species) reported in various countries of the Mediterranean Basin (Castillo *et al.* 2010, Ali *et al.* 2014, Tzortzakakis *et al.* 2014, 2015, Archidona-Yuste *et al.* 2016b, a). The present results increase the previous data about diversity of *Xiphinema* species detected in olive worldwide, including four new species from the *X.* non-*americanum*-group. All these species were new records for olive with the exception of *X. pachtaicum*, *X. index*, *X. italiae*, *X. nuragicum* and *X. turcicum* (Ali *et al.* 2014). In addition to the remarkable prevalence of *Xiphinema* spp. observed in both olive types, our study showed a great species diversity, that was mainly associated with the *X.* non-*americanum*-group species ($P < 0.05$, Figure 5.2D), being widely distributed in Andalusia but in particular mainly associated with wild olive in Cádiz province, a more humid and ecologically diverse area than the rest of the Andalusian provinces. However, *X. pachtaicum* was present in the majority of the sampled localities in wild and cultivated olives showing the plasticity of this species for a wide diversity of ecological conditions (Figure 5.1). Overall, *X. pachtaicum* was detected in 74.2% of the total sampling sites, specifically 67 out of 115 and 268 out of 338 associated with wild and cultivated olive, respectively. As reported in previous studies, this species is widespread in the Mediterranean Basin (Taylor and Brown 1997, Téliz *et al.* 2007, Gutiérrez-Gutiérrez *et al.* 2011a, 2011b, 2012, Tzortzakakis *et al.* 2014, Archidona-Yuste *et al.* 2016a, Guesmi-Mzoughi *et al.* 2017), including olive (Hashim 1979, Palomares-Rius *et al.* 2014, Palomares-Rius *et al.* 2015). In Spain, *X. pachtaicum* was also the most prevalent dagger nematode in vineyards and stone-fruit orchards (Arias and Navacerrada 1973, Gutiérrez-Gutiérrez *et al.* 2011b). The widespread distribution of *X. pachtaicum* may suggest also adaptability to a range of soil types, and reproduction sustained over a broad range of temperatures (Navas *et al.* 1988, Brown *et al.* 1994). Nevertheless, these wider ecological requirements are difficult to explain regarding their low genetic diversity (Gutiérrez-Gutiérrez *et al.* 2011a) and could be more associated with the presence of specific ovarial-intestine endosymbionts (Palomares-Rius *et al.* 2016), but some of the other species from the *X. americanum*-group also possesses ovarial-intestine endosymbionts and were more restricted to some areas (*viz. X.*

opisthohysterum, *X. santos*, *X. incertum*, *X. madeirense*, *X. vallense*, *X. plesiopachtaicum* and *X. rivesi*) (Palomares-Rius *et al.* 2016). Other species with a broad distribution were included in the *X.* non-*americanum*-group, i.e. *X. italiae* found in all provinces, *X. nuragicum* in 7 out of 8 provinces, and *X. coxi europaeum* in 5 out of 8 provinces. In this sense, the presence of a high number of frequent species belonging to *X.* non-*americanum*-group (i.e. *X. italiae*, *X. nuragicum*, *X. coxi europaeum* or to a lesser extent *X. adenohystherum*) explains the higher value observed in Hill´s 2 (Dominance diversity) index with respect to *X. americanum*-group ($P < 0.01$, Figure 5.2D).

Nematodes of the genus *Xiphinema* cause damage to olive by feeding on unmodified plant root cells and causing cell necrosis and galling in root apex (Sasanelli *et al.* 1999, Castillo *et al.* 2003). However, some species are also capable to transmit pathogenic viruses to olive, specifically species belonging to the *Nepovirus* genus (Decraemer and Robbins 2007), such as *X. diversicaudatum* and *X. vuittenezi* (Martelli and Taylor 1990). Nevertheless, some dagger nematodes have been considered as major pathogens on olive trees in several countries including Chile or USA, where it was reported that *Xiphinema* spp. were responsible for 5 to 10% of loss production resulting in an estimated $39 million loss (Hashim 1983, Koenning *et al.* 1999). Although our results mainly revealed low densities of *Xiphinema* spp. in both olive types studied (Tables 5.2 and S5.10), in some sampling sites the densities were high, i.e. 414 or 350 nematodes per 500 cm^3 of soil for *X. pachtaicum* and *X. italiae*, respectively. In this regard, similar nematode densities of *Xiphinema* spp. have been reported to reduce plant-growth by feeding directly on olive roots, e.g. 65% in the case of *X. elongatum* (Lamberti and Vovlas 1993), and in several plants including other crops (McElroy 1972, Lal *et al.* 1982) or ornamental plants (Schindler 1957). On the other hand, total abundance of nematodes in each sampling site resulted significantly higher in *X. americanum*-group in comparison to *X.* non-*americanum*-group ($P < 0.001$, Figure 5.2D). We found a significant increase in the abundance in cultivated than in wild olive ($P < 0.01$, Figure 5.2B) for the *X. americanum*-group, mainly because of the prevalence and high average nematode density detected for *X. pachtaicum* on cultivated olive (Tables 5.2 and S5.10). Overall, these results could support the hypothesis that *X. pachtaicum* could be a real problem in olive orchards (Peña-Santiago 1990), although more studies would be required to clarify it. In general, *Xiphinema* spp. are difficult to culture under

glasshouse conditions, and it is possible, that these nematodes are more pathogenic to olive in the field than is indicated by glasshouse test, since their population densities in such situations are likely to exceed those that can be attained in glasshouses (Hashim 1983).

Overall, nematode diversity decreases rapidly to agricultural management including plant-parasitic nematodes (Yeates and Bongers 1999). Our results showed lower diversity indexes values, specifically for Richness diversity, in wild than in cultivated olives ($P < 0.001$, Figure 5.2A). These differences were emphasized when *X. americanum*-group and *X.* non-*americanum*-group species were analysed separately ($P < 0.05$, Figures 5.2B and 5.2C). This fact showed the effect of agricultural management to a wide range of changes in physical, chemical and biological properties of the soil, and alterations in the autoregulation in nematode assemblages, when compared natural (wild olive) with agricultural ecosystems (cultivated olive). In this sense, several papers showed the effect of these parameters or agricultural practices in the olive nematode community (Palomares-Rius *et al.* 2012, 2015, Sánchez-Moreno *et al.* 2015). However, according to the higher number of species identified from *X.* non-*americanum*-group likewise the high prevalence of this group of nematodes associated to wild olive resulted in a higher value of Richness diversity in this type of olive in comparison to cultivated olive ($P < 0.05$, Table 5.2 and Figure 5.2C) in contrast to observed in *X. americanum*-group showing the possible plasticity of this species for a wide diversity of ecological requirements as discussed above. On the other hand, the distribution of the 385 *Xiphinema* populations collected in Andalusia did not revealed geographic associations to certain areas (Figure 5.1). Although agricultural activities may result in the widespread dissemination of *Xiphinema* species (Brown *et al.* 1994), the geographical distribution of *Xiphinema* species in wild and cultivated olives in southern Spain suggest a pattern linked to ecological factors. As previously reported by Archidona-Yuste *et al*. (2016c) for *Longidorus* species: “longidorids could have a lower dissemination level by human activities than other plant-parasitic nematodes (i.e. cyst- or root-lesion nematodes) because of their sensitivity to fast desiccation, large body size, and the absence of survival-resistance forms”. Unfortunately, little is known about the ecological requirements of *Xiphinema* nematodes and further research is needed (Brown *et al.* 1994).

Some provinces as Cádiz, Córdoba and Jaén have showed a higher diversity than other with 17, 15 and 12 species, respectively. Some of these provinces as Cádiz showed more favourable environment for nematodes development due to the higher relative humidity and water content in the soil. By contrast, *Longidorus* spp. showed evidence of some geographic species associations in Andalusia (Archidona-Yuste *et al.* 2016c). Consequently, further research is needed in order to determine the influence of physico-chemical soil factors on the prevalence and distribution of *Xiphinema* spp. in southern Spain and other wider areas.

Sequences of nuclear ribosomal RNA genes, particularly D2-D3 and ITS1, are useful molecular markers for providing accurate species identification of Longidoridae (He *et al.* 2005, Palomares-Rius *et al.* 2008, Gutiérrez-Gutiérrez *et al.* 2012, Archidona-Yuste *et al.* 2016a, b, c). The majority of the identified species in the rhizosphere of olive matched former molecularly characterized species in other studies. In this sense, this study provides new molecular markers for partial 18S (*X. cadavalense*, *X. pseudocoxi*, and *X. conurum*) and for ITS1 (*X. cadavalense*, *X. pseudocoxi*, *X. cohni*, *X. opisthohysterum* and *X. duriense*). D2-D3 expansion region was more useful for establishing phylogenetic relationships among *Xiphinema* species than ITS1 or 18S. Phylogenetic analyses based on D2-D3, ITS1, and partial 18S using BI resulted in a consistent position for the newly described species of *X.* non-*americanum*-group species from Spain, which grouped in two separated clades, and mostly agree with the clustering obtained by other authors (Gutiérrez-Gutiérrez *et al.* 2013b, Archidona-Yuste *et al.* 2016b). These species showed a good congruence between morphometric characters and phylogenetic positions as it is the case of *X. andalusiense* sp. nov., *X. baetica*, and *X. cadavalense*. In the case of *X. andalusiense* sp. nov. vs *X. baetica*, only lower a and c' ratios, the absence of spines in the uterus, the absence of males and different ribosomal genes could separate *X. baetica* from *X. andalusiense* sp. nov. These species probably evolved in the Iberian Peninsula as they occur only there. The Iberian Peninsula has been suggested as a possible center of recent speciation for PPN nematode genera such as *Longidorus*, *Trichodorus* or *Rotylenchus* species (Tzortzakakis *et al.* 2014). *Xiphinema celtiense* sp. nov., *X. iznajarense* sp. nov. and *X. mengibarense* sp. nov. could be clearly separated morphologically and molecularly from the other *Xiphinema* species. The majority of the species showed congruence in the phylogenetic relationships within D2-D3, ITS1, and partial 18S using the

DNA from the same individual and these markers matched very well with the sequences deposited in the GenBank. This result is in contrast with the close related genus Longidorus found in a similar sampling scheme and localities in which the diversity of species was lower and all the species occupies two major positions in the phylogenetic clade (Archidona-Yuste *et al.* 2016a).

5. Conclusions

In summary, this study provides new insights into the diversity of this genus associated with the olive in Mediterranean conditions with important differences related to the species within the *X. americanum*-group and the *X.* non-*americanum*-group species. This research provides molecular markers for precise and unequivocal diagnosis of some species of *Xiphinema* in order to differentiate virus vector or quarantine species. Furthermore, it reflects that similar intensive and extensive integrative studies on *Xiphinema* species based on widest areas may help to elucidate the evolutionary origin of *Xiphinema* species. In this sense, further studies based on widespread species (i.e. *X. pachtaicum*) could also help to clarify if the main speciation occurred in Africa leading to many apomictic species in tropical and subtropical environments as hypothesised by Coomans (1985), or in South America but in this case information is limited.

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8. Supplementary Information

Table 5.10 Average soil nematode population density (number of specimens) and prevalence (%) of *Xiphinema* spp. in wild and cultivated olives in provinces of Andalusia, southern Spain.[a]

	Andalusian provinces															
	Almería		**Cádiz**		**Córdoba**		**Granada**		**Huelva**		**Jaén**		**Málaga**		**Seville**	
Host plant[b]	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**
Number of samples	8	25	53	19	23	79	1	39	9	20	6	63	8	28	7	65
***Xiphinema* spp.**	**12.6 (50.0)**	**36.6 (52.0)**	**23.3 (100)**	**39.3 (100)**	**25.5 (95.7)**	**38.0 (83.4)**	**11.0 (100)**	**33.2 (84.6)**	**19.4 (100)**	**47.2 (95.0)**	**28.6 (100)**	**32.5 (79.4)**	**20.5 (87.5)**	**42.0 (85.7)**	**15.2 (85.7)**	**39.2 (81.5)**
***X. americanum*-group spp.[c]**	**15.0 (50.0)**	**45.4 (52.0)**	**19.4 (83.0)**	**49.9 (100)**	**27.3 (78.3)**	**44.3 (81.0)**	**11.0 (100)**	**31 (79.5)**	**18.6 (55.6)**	**48.3 (90.0)**	**45.6 (83.3)**	**35.4 (77.8)**	**24.9 (87.5)**	**43.6 (82.1)**	**22.2 (85.7)**	**47.0 (81.5)**
Xiphinema duriense	-	-	-	-	-	-	-	-	2.0 (11.1)	1.0 (5.0)	-	-	-	-	-	-
Xiphinema incertum	-	-	17.9 (13.2)	-	38.5 (8.7)	-	-	-	28.0 (11.1)	-	-	-	22.0 (12.5)	-	-	38.0 (1.5)
Xiphinema madeirense	-	-	11.0 (1.9)	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiphinema opisthohysterum	-	-	3.0 (1.9)	-	-	-	-	-	-	-	14.0 (16.1)	-	-	-	-	-
Xiphinema pachtaicum	15.0 (50.0)	45.4 (52.0)	20.7 (52.8)	54.1 (100)	25.9 (69.6)	44.8 (81.0)	11.0 (100)	38.7 (79.5)	21.0 (33.3)	53.2 (85.0)	43.0 (33.3)	35.4 (77.8)	25.3 (87.5)	43.6 (82.1)	2.2 (85.7)	47.2 (81.5)
Xiphinema parapachydermum	-	-	31.8 (7.5)	-	-	8.0 (1.3)	-	-	-	-	16.0 (16.1)	-	-	-	-	-
Xiphinema plesiopachtaicum	-	-	-	-	-	-	-	-	-	-	112 (16.1)	-	-	-	-	-
Xiphinema santos	-	-	9.0 (1.9)	-	-	-	-	-	-	-	-	-	-	-	-	-

	Andalusian provinces															
	Almería		Cádiz		Córdoba		Granada		Huelva		Jaén		Málaga		Seville	
Host plant[b]	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**
Xiphinema rivesi	-	-	-	-	-	-	-	-	-	58.0 (5.0)	-	-	-	-	-	-
Xiphinema vallense	-	-	13.6 (13.2)	14.0 (10.5)	-	-	-	-	-	-	-	-	-	-	-	-
***X. non-americanum-group spp.*[c]**	**3 (12.5)**	**13.8 (20.0)**	**26.7 (84.9)**	**16.9 (52.6)**	**23.9 (73.9)**	**16.4 (21.5)**	**-**	**4.7 (15.4)**	**19.8 (88.9)**	**42.8 (30.0)**	**11.6 (66.7)**	**8.3 (9.5)**	**8.7 (37.5)**	**38.8 (39.3)**	**4.8 (42.9)**	**23.0 (36.9)**
***Xiphinema andalusiense* sp. nov.**	-	-	-	-	12.5 (8.7)	-	-	-	-	-	16.0 (16.1)	-	-	-	-	-
Xiphinema celtiense sp. nov.	-	-	-	-	82.0 (4.4)	-	-	-	-	-	-	-	-	-	3.0 (14.3)	-
Xiphinema iznajarense sp. nov.	-	-	-	-	-	34.0 (1.3)	-	-	-	-	-	-	-	-	-	-
***Xiphinema mengibarense* sp. nov.**	-	-	-	-	-	-	-	-	-	-	-	12.0 (1.6)	-	-	-	-
Xiphinema adenohystherum	-	-	3.3 (15.1)	-	10.3 (17.4)	1.0 (1.3)	-	-	-	-	14.0 (16.7)	-	-	-	-	-
Xiphinema baetica	-	-	1 (1.9)	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiphinema cadavalense	-	-	-	-	-	1.0 (1.3)	-	-	-	-	-	-	-	-	-	-
Xiphinema cohni	-	-	32 (1.9)	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiphinema conurum	-	3 (7.70)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiphinema coxi europaeum	-	-	10.0 (3.8)	-	1.3 (13.0)	1.0 (2.5)	-	-	31.7 (33.3)	-	-	-	-	-	10.0 (14.3)	-
Xiphinema hispanum	-	-	-	-	-	-	-	-	-	-	6.5 (33.3)	-	-	-	-	-

Andalusian provinces

	Almería		Cádiz		Córdoba		Granada		Huelva		Jaén		Málaga		Seville	
Host plant[b]	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**	**W**	**C**
Xiphinema hispidum	-	-	5.3 (7.6)	-	-	-	-	-	12.0 (11.1)	-	-	-	-	-	-	-
Xiphinema index	-	-	-	-	-	3.0 (1.3)	-	-	-	-	-	-	-	-	-	-
Xiphinema italiae	3 (12.5)	16.5 (16.0)	60.4 (17.0)	13.6 (26.3)	-	7.7 (3.8)	-	3.0 (5.1)	20.5 (22.2)	51.5 (20.0)	-	13.5 (3.2)	9.0 (12.5)	59.0 (3.6)	-	19.5 (21.5)
Xiphinema lupini	-	-	-	-	-	-	-	-	8.0 (22.2)	-	-	-	-	-	4.0 (14.3)	-
Xiphinema macrodora	-	-	-	-	7.0 (4.4)	14.0 (1.3)	-	-	-	8.0 (5.0)	-	-	-	-	-	-
Xiphinema nuragicum	-	-	35.7 (49.1)	23.0 (21.1)	40.4 (30.4)	25.9 (11.4)	-	6.3 (2.6)	14.0 (11.1)	-	-	2.5 (3.2)	8.5 (25.0)	38.4 (32.1)	-	29.4 (16.9)
Xiphinema oleae	-	-	4 (1.9)	-	-	-	-	-	-	-	-	-	-	-	-	-
Xiphinema pseudocoxi	-	-	-	-	10.0 (4.4)	-	-	-	-	-	-	-	-	-	-	-
Xiphinema sphaerocephalum	-	-	-	-	-	-	-	-	-	-	15.0 (16.1)	-	-	-	-	-
Xiphinema turcicum	-	-	2.3 (5.7)	15.0 (5.3)	-	-	-	3.0 (2.6)	-	-	-	6.0 (1.6)	-	22.0 (3.6)	2.0 (14.3)	1.0 (1.5)
Xiphinema turdetanense	-	-	2.2 (9.4)	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Population density was calculated as the mean of *Xiphinema* nematodes per 500 cm^3 of soil. The prevalence was computed by dividing the numbers of samples in which the *Xiphinema* species was observed by the total number of samples and expressed as a percentage

[b] Host plant: W = wild olive; C = cultivated olive.

[c] *Xiphinema* group species established by Loof and Luc 1990; Lamberti *et al.* 2000; and Coomans *et al*. 2001 (-) not found

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Unravelling the biodiversity and molecular phylogeny needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) in olive and a description of six new species

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Abstract

The genus *Longidorus* includes a remarkable group of invertebrate animals of the phylum Nematoda comprising polyphagous root-ectoparasites of numerous plants including several agricultural crops and trees. Damage is caused by direct feeding on root cells as well as by transmitting nepoviruses that cause disease on those crops. Thus, correct identification of *Longidorus* species is essential to establish appropriate control measures. We provide the first detailed information on the diversity and distribution of *Longidorus* species infesting wild and cultivated olive soils in a wide-region in southern Spain that included 159 locations from which 449 sampling sites were analyzed. The present study doubles the known biodiversity of *Longidorus* species identified in olives by including six new species (***Longidorus indalus* sp. nov., *Longidorus macrodorus* sp. nov., *Longidorus onubensis* sp. nov., *Longidorus silvestris* sp. nov., *Longidorus vallensis* sp. nov., and *Longidorus wicuolea* sp. nov.**), two new records for wild and cultivate olives (*L. alvegus* and *L. vineacola*), and two additional new records for wild olive *(L. intermedius* and *L. lusitanicus*). We also found evidence of some geographic species associations to western (*viz. L. alvegus*, *L. intermedius, L. lusitanicus*, *L. onubensis* sp. nov., *L. vineacola*, *L. vinearum*, *L. wicuolea* sp. nov.) and eastern distributions (*viz. L. indalus* sp. nov.), while only *L. magnus* was detected in both areas. We developed a comparative study by considering morphological and morphometrical features together with molecular data from nuclear ribosomal RNA genes (D2–D3 expansion segments of 28S, ITS1, and partial 18S). Results of molecular and phylogenetic analyses confirmed the morphological hypotheses and allowed the delimitation and discrimination of six new species of the genus described herein and four known species. Phylogenetic analyses of *Longidorus* spp. based on three molecular markers resulted in a general consensus of these species groups, since lineages were maintained for the majority of species. This study represents the most complete phylogenetic analysis for *Longidorus* species to date.

1. Introduction

The phylum Nematoda comprises the most species-rich metazoans on earth with a global distribution and estimated realistic number of species of *ca*. 105 (Boucher and Lambshead 1995, Blaxter *et al.* 1998, Coomans 2000). Soil nematode gross morphology tends to be highly conserved, making species identification a very difficult task (Coomans 2000, Siddiqi 2000). Accurate diagnostic studies of plant-parasitic nematode (PPN) species are important because of their implications in pest control and soil ecology (Coomans *et al.* 2001). With most nematode species likely remaining undescribed, efforts to catalogue and explain biodiversity need to be prioritized (Bickford *et al.* 2007). However species concept ranges among typological species (a community of specimens described by characteristic features of its type specimen), biological species (populations which successfully interbreed with each other), and phylogenetic species (phylogenetic lineages). All of these concepts have limitations, including the popular biological species concept which is restricted to sexual, outcrossing populations and excludes parthenogenetic organisms (Subbotin and Moens 2006, Palomares-Rius *et al.* 2014). Species delimitation in nematodes typically uses a phenotypic view of the animal, based in relatively few anatomical and morphological characters, such as lip region and female tail shape, pharyngeal glands, stylet shape and length, type of female reproductive system, etc. Additionally, many nematodes have complex life-cycles and it can be difficult to demonstrate the validity of a species by means of intercrossing of individuals and production of viable progeny. For these reasons the possibility of undescribed or misdescribed species is very high, as demonstrated by several authors (Oliveira *et al.* 2006, Gutiérrez-Gutiérrez *et al.* 2010, Cantalapiedra-Navarrete *et al.* 2013, Palomares-Rius *et al.* 2014).

The family Longidoridae Thorne, 1935 includes a wide and diverse group of migratory ectoparasitic nematode species, where the needle nematodes of the genus *Longidorus* Micoletzky, 1922 is one of the most evolved group species of this family (Coomans 1996). This genus includes a number of long to very long body (2–12 mm) specimens with long stylet (80–260 µm). They are polyphagous species of many plants including various agricultural crops, and cause damage by direct feeding on root cells

as well as by transmitting nepoviruses (nepoviruses are spherical, single-stranded RNA of positive-sense) (Taylor and Brown 1997, Macfarlane *et al.* 2002, Macfarlane 2003). Some *Longidorus* spp. are cosmopolitan whilst others have a limited geographic distribution (Coomans 1996). The genus *Longidorus* is a diverse group with about 160 nominal species (Gutiérrez-Gutiérrez *et al.* 2013, Peneva *et al.* 2013), but only 11 species (6.9%) (*L. apulus*, *L. arthensis*, *L. attenuatus*, *L. caespiticola*, *L. diadecturus*, *L. elongatus*, *L. fasciatus*, *L. leptocephalus*, *L. macrosoma*, *L. martini*, and *L. profundorum*) have been reported as virus vector, but transmitting seven out of the 38 known nepoviruses (Taylor and Brown 1997, Decraemer and Robbins 2007). Nepoviruses vectored by *Longidorus* species damage vegetable and fruit crops including: *Artichoke Italian latent virus*, *Cherry rosette disease virus*, *Tomato black ring virus*, *Raspberry ringspot virus*, *Arabis mosaic virus*, Peach rosette mosaic virus, and *Mulberry ringspot virus* (Taylor and Brown 1997, Decraemer and Robbins 2007). Therefore, correct identification of *Longidorus* species is essential to establish appropriate control measures. Species discrimination in *Longidorus* has classically been based mainly on morphology and morphometrics of diagnostic features. However morphologically based species characterization is complicated by a high degree of intraspecific variability within morphometrics, as well as slight interspecific differences that lead to substantial overlapping among *Longidorus* species and increase the risk of species miss-identification (Gutiérrez-Gutiérrez *et al.* 2010, 2013). As a result, taxonomic difficulties often arise from under- or over-estimation of intraspecific variability of certain morphological characters currently being used for species diagnosis.

Integrative taxonomy assembles and assimilates all available data and information to frame species limits (phenotypic, genotypic and phylogenetic) (Subbotin and Moens 2006, Palomares-Rius *et al.* 2014). Although this approach is more complex and has a higher cost than traditional taxonomy, its application reduce the degree of subjectivity that is common in traditional alpha taxonomic practices, as has been recently reported in studies showing the potential for these methods in the discovery of new and cryptic species in taxa poorly known or composed of morphologically conserved species (Ye *et al.* 2004, Bickford *et al.* 2007, Gutiérrez-Gutiérrez *et al.* 2010, 2012, 2013, Palomares-Rius *et al.* 2014).

Recently, 68 *Longidorus* species (about 42% of total species) have been characterized molecularly, constituting a useful tool for molecular-based species identification. Molecular approaches using multiple regions of the ribosomal DNA (rDNA) genes sequences including (28S, 18S, and 5.8S genes and internal transcribed spacers (ITS1 and ITS2)), have been investigated to better understand the taxonomic relationships within the genus *Longidorus* (De Luca *et al.* 2004, Neilson *et al.* 2004, Ye *et al.* 2004, He *et al.* 2005, Palomares-Rius *et al.* 2008, De Luca *et al.* 2009, Gutiérrez-Gutiérrez *et al.* 2011, 2013). These molecular markers have been shown to be useful diagnostic tools in the characterization and phylogenetic relationships within Longidoridae, particularly in cases where morphological characters may lead to ambiguous interpretation, such as species in the *Xiphinema americanum*-group (De Luca *et al.* 2004, Neilson *et al.* 2004, Ye *et al.* 2004, He *et al.* 2005, Palomares-Rius *et al.* 2008, De Luca *et al.* 2009, Gutiérrez-Gutiérrez *et al.* 2011, 2013). D2–D3 expansion segments of 28S rRNA and ITS1 rRNA have proven to be a powerful tool for providing accurate and molecular species identification in Longidoridae compared to partial 18S, since both molecular markers showed more species variability (nucleotides and indels) than partial 18S, which in some cases did not show enough resolution to distinguish species (Neilson *et al.* 2004, He *et al.* 2005, Pedram *et al.* 2008, Gutiérrez-Gutiérrez *et al.* 2011, 2013).

Longidorus species identification remains quite challenging when dealing with species that closely resemble one another and which co-occur in a region, as is often the case in the Iberian Peninsula. Furthermore, soil samples often contain mixed populations with more than one species in the same sample. In this study we focus mostly on the *Longidorus* species that occur throughout wild and cultivate olives at southern Spain. Morphological and morphometric evaluation as well as molecular sequencing of each Longidorus population were used simultaneously for species delineation and grouping specimens into species.

Olive, the emblematic tree of the Mediterranean Basin, is found in two forms, namely wild (*Olea europaea* subsp. *europaea* var. *sylvestris*) and cultivated (*Olea europaea* subsp. *Europaea* var. *europaea*) (Belaj *et al.* 2007). Wild olives occur throughout many Mediterranean environments, characterized by semi-arid climatic conditions with different altitudes, plant communities and soils, including those with extreme dry conditions (Belaj *et*

al. 2007). Cultivated olive is extensively grown in the Mediterranean Basin, as well as the subtropical regions of Australia, southern Africa, and North and South America (Zohary and Spiegel-Roy 1975). Olive is the most cultivated non-tropical fruit trees and is among the most ancient crops in the Mediterranean Basin (Zohary and Spiegel-Roy 1975). Approximately 10.5 million ha of cultivate olive are growing in the world, of which about 85% are in Mediterranean countries, including North Africa, and about 25% of them in Spain (FAOSTAT 2014). In Andalusia, southern Spain, cultivated olive trees cover more than 1.6 million ha accounting for 19% of the total surface area in an impressive monoculture (CAP-JA 2014, MAGRAMA 2014).

Both wild and cultivated olive trees serve as hosts to a large number of plant-parasitic nematodes, of which root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.), spiral nematodes (*Helicotylenchus* spp.), and needle and dagger nematodes (*Longidorus* spp., *Xiphinema* spp.) are widely distributed and damage this crop (Castillo *et al.* 2010, Ali *et al.* 2014). However, little information is available about needle nematodes associated with olive trees, except for the recent contribution of Palomares-Rius et al. (Palomares-Rius *et al.* 2015) reporting *Longidorus magnus* Lamberti, Bleve-Zacheo and Arias 1982 (Lamberti *et al.* 1982) and *Longidorus* sp. According to Gutiérrez-Gutiérrez et al. (Gutiérrez-Gutiérrez *et al.* 2013) and other authors, 30 species of the genus Longidorus have been reported in Spain, mainly associated with fruit, forest, ornamental and vegetable plant species (Peña Santiago *et al.* 2003, Palomares-Rius *et al.* 2010, Gutiérrez-Gutiérrez *et al.* 2011, 2013).

With the aim of deciphering the biodiversity of *Longidorus* spp. infecting wild and cultivated olives in southern Spain, we sampled a total of 159 nine localities at the eight provinces of Andalusia where both olive types were present. In this survey we detected 40 populations of *Longidorus* species characterized by moderate to large body and stylet length, apparently morphologically related to other known *Longidorus* spp. This prompted us to carry out an integrative taxonomic study to assess the power of this approach for species identification within this complex genus.

The overall objective of this study was to test the congruence between morphological and molecular data within Longidorus species, and the specific objectives were: i) to identify and morphologically and morphometrically compare the 40 Spanish populations of *Longidorus* spp. detected in recent field samples from wild and cultivate olive-ecosystems; ii) to carry out a molecular characterisation of these Longidorus populations based on sequences of the D2–D3 expansion segments of the 28S nuclear ribosomal RNA gene, the ITS1 of rRNA, and partial 18S rRNA sequences; and iii) to study the phylogenetic relationships of *Longidorus* spp.

2. Material and Methods

2.1 Ethics Statement

No specific permits were required for the described fieldwork studies. Permission for sampling the olive orchards was granted by the landowner. The samples from wild olives were obtained in public areas, forests, and other natural areas studied and do not involve any species endangered or protected in Spain. The sites are not protected in any way.

2.2 Soil collection and nematode extraction

Nematodes were surveyed from 2012 to 2015 during the spring season in wild and cultivate olives groves in Andalusia, southern Spain (Table 6.1, Figure 6.1). Soil samples were collected for nematode analysis with a shovel from four to five trees in each sampling site. A total of 131 and 318 sampling sites from wild and cultivated olives, respectively, were arbitrarily chosen in the eight provinces of Andalusia where both olive types were present. The number of sampling sites was proportional to the area of wild and cultivated olive in each province (Table 6.1, Figure 6.1). Soil samples were collected from a 5- to 50-cm depth, in the close vicinity of active plant roots, discarding the upper 5-cm of topsoil to ensure that roots from weeds or other herbaceous plants were not included. All soil samples from each site were thoroughly mixed to obtain a single representative sample before nematode extraction.

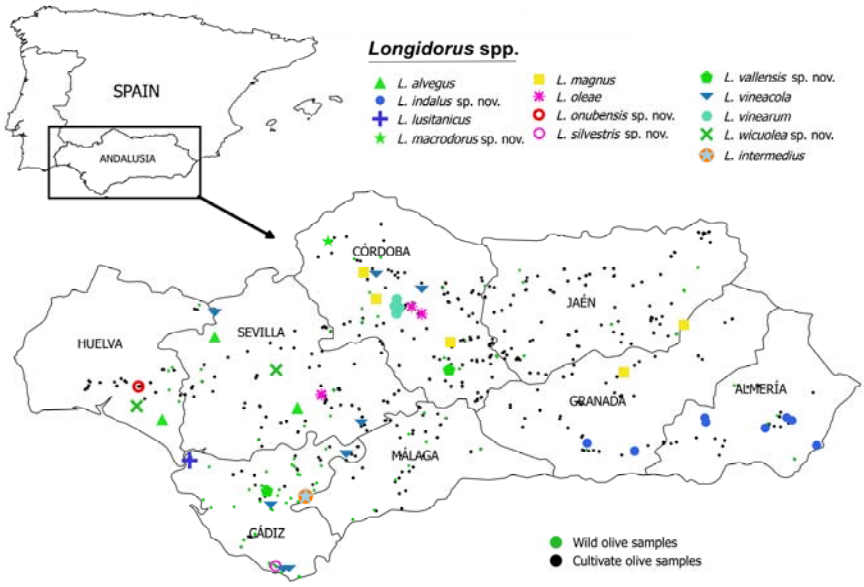

Figura 6.1: Geographic distribution of needle nematodes of the genus *Longidorus* in the present fieldworks on wild and cultivated olive in southern Spain. This map may be similar but not identical to other published maps of Andalusia and is therefore for illustrative purposes only on the sampling sites.

Nematodes were extracted from a 500-cm^3 sub-sample of soil using magnesium sulphate centrifugal-flotation and a modification of Cobb´s decanting and sieving methods (Flegg 1966, Coolen 1979). The soil was washed thoroughly with tap water through a 710-µm mesh sieve, and the filtered water was collected in a beaker and thoroughly mixed with 4% kaolin (v/v). This mixture was centrifuged at 1,100×g for 4 min, and the supernatants discarded. Pellets were resuspended in 250 ml MgSO4 (δ = 1.16) and the new suspensions were centrifuged at 1,100×g for 3 min. The supernatants were sieved through a 5 µm mesh, and nematodes collected on the sieve were washed with tap water (Coolen 1979). The nematode sample was poured into a counting dish (8 cm L × 8 cmW× 1.5 cm H) and the nematodes were identified and counted under a Leica MZ12, stereomicroscope (Leica Microsystems, Wetzler, Germany). PPN from soil samples were identified to genus, and then we focussed on the species

delineation of needle nematodes of the genus *Longidorus*. Later on, abundance and prevalence of each *Longidorus* species was estimated. Abundance was calculated as the mean number of *Longidorus* nematodes per 500 cm^3 of soil for all samples. The prevalence was computed by dividing the number of samples in which the *Longidorus* species was detected by the total number of samples and expressed as a percentage.

2.3 Morphological studies

Longidorus specimens for light microscopy were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerine using Seinhorst's method (Seinhorst 1962). Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at up to 1,000x magnification. The morphometric study of each nematode population included classical diagnostic features in longidoridae (i.e. de Man body ratios, lip region and amphid shape, oral aperture-guiding ring, odontostyle and odontophore length) (Jairajpuri and Ahmad 1992). All measurements were expressed in micrometers (µm), unless otherwise indicated in text. For line drawing of the new species, light micrographs were imported to CorelDraw software version X6 (Corel Corporation, London, UK) and redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992). In addition, a comparative morphological and morphometrical study of type specimens of some species were conducted with specimens kindly provided by Dr. A. Troccoli, from the nematode collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (*viz. Longidorus lusitanicus* Macara 1985, *Longidorus vinearum* Bravo and Roca 1995), and Dr. A. Navas from the Nematode Collection of the Spanish National Museum of Natural Sciences-CSIC, Madrid, Spain (*viz. Longidorus carpetanensis* Arias, Andrés and Navas 1986 and *Longidorus unedoi* Arias, Andrés and Navas 1986).

Table 6.1 Taxa sampled for *Longidorus* species and sequences used in this study.

Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
1. ***Longidorus indalus* sp. nov.**	ST041	Las Tres Villas (Almería, Spain)	culti. live	KT308852	KT308878	KT308894
2. ***Longidorus indalus* sp. nov.**	AR046	Agua Amarga (Almería, Spain)	wild olive	KT308853	-	KT308895
3. ***Longidorus indalus* sp. nov.**	ST193	Lecrín (Granada, Spain)	culti. live	KT308854	KT308879	-
4. ***Longidorus indalus* sp. nov.**	ST042	Las Tres Villas (Almería, Spain)	culti. live	*	-	-
5. ***Longidorus indalus* sp. nov.**	AR044	Sorbas (Almería, Spain)	wild olive	*	-	-
6. ***Longidorus indalus* sp. nov.**	ST045	Sorbas (Almería, Spain)	culti. live	*	-	-
7. ***Longidorus indalus* sp. nov.**	JAO66	Lobras (Granada, Spain)	culti. live	*	-	-
8. ***Longidorus indalus* sp. nov.**	JAO73	Tabernas (Almería, Spain)	culti. live	*	-	-
9. ***Longidorus macrodorus* sp. nov.**	JAO06	La Grajuela (Córdoba, Spain)	culti. live	KT308855-KT308856	KT308880-KT308881	KT308896
10. ***Longidorus onubensis* sp. nov.**	ST005	Niebla (Huelva, Spain)	culti. live	KT308857-KT308858	KT308882-KT308883	KT308897
11. ***Longidorus silvestris* sp. nov.**	AR027	Tarifa (Cádiz, Spain)	wild olive	KT308859-KT308860	KT308884	KT308898
12. ***Longidorus vallensis* sp. nov.**	AR055	San José del Valle (Cádiz, Spain)	wild olive	KT308861	KT308885	KT308899
13. ***Longidorus vallensis* sp. nov.**	M0012	Cabra (Córdoba, Spain)	culti. live	KT308862	KT308886	-
14. ***Longidorus wicuolea* sp. nov.**	JAO95	Carmona (Sevilla, Spain)	culti. live	KT308863-KT308864	KT308887	KT308900
15. ***Longidorus wicuolea* sp. nov.**	AR101	Bonares (Huelva, Spain)	wild olive	KT308865-KT308866	KT308888-KT308889	-
16. ***L. alvegus* Roca *et al*. 1989**	JAO107	Utrera (Sevilla, Spain)	culti. live	KT308867		

	Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
17.	***L. alvegus* Roca *et al*. 1989**	AR110	Almadén de la Plata (Sevilla, Spain)	wild olive	*	-	-
18.	***L. alvegus* Roca *et al*. 1989**	AR099	El Rocío (Huelva, Spain)	wild olive	*	-	-
19.	***L. intermedius* Kozlowska and Seinhorst 1979**	AR131	Jerez de la Frontera (Cádiz, Spain)	wild olive	KT308868	KT308890	-
20.	***L. lusitanicus* Macara 1986**	J212B	Sanlúcar de Barrameda (Cádiz, Spain)	wild olive	KT308869	KT308891	KT308901
21.	***L. magnus* Lamberti *et al*. 1982**	ST146	Castril (Granada, Spain)	culti. live	KT308870	-	KT308902
22.	***L. magnus* Lamberti *et al*. 1982**	ST077	Espiel (Córdoba, Spain)	culti. live	*	-	-
23.	***L. magnus* Lamberti *et al*. 1982**	ST203	Morelábor (Granada, Spain)	culti. live	*	-	-
24.	***L. magnus* Lamberti *et al*., 1982**	JAO01	Villaviciosa (Córdoba, Spain)	culti. live	*	-	-
25.	***L. oleae* Gutiérrez-Gutiérrez *et al*. 2013**	AR112	Córdoba (Córdoba, Spain)	wild olive	KT308871	-	-
26.	***L. oleae* Gutiérrez-Gutiérrez *et al.* 2013**	AR113	Córdoba (Córdoba, Spain)	wild olive	*	-	-
27.	***L. oleae* Gutiérrez-Gutiérrez *et al.* 2013**	AR024	Marchena (Sevilla, Spain)	wild olive	*	-	-
28.	***L. oleae* Gutiérrez-Gutiérrez *et al*. 2013**	OL057	Marchena (Sevilla, Spain)	culti. live	*	-	-
29.	***L.vineacola* Sturhan and Weischer 1954**	ST016	El Saucejo (Sevilla, Spain)	culti. live	KT308872	-	-
30.	***L.vineacola* Sturhan and Weischer 1954**	AR031	Tarifa (Cádiz, Spain)	wild olive	KT308873	-	-
31.	***L.vineacola* Sturhan and Weischer 1954**	AR006	Alcalá de los Gazules (Cádiz, Spain)	wild olive	*	-	-
32.	***L.vineacola* Sturhan and Weischer 1954**	AR032	Tarifa (Cádiz, Spain)	wild olive	*	-	-
33.	***L.vineacola* Sturhan and Weischer 1954**	ST117	Setenil de las Bodegas (Cádiz, Spain)	culti. live	*	-	-
34.	***L.vineacola* Sturhan and Weischer 1954**	AR110	Almadén de la Plata (Huelva, Spain)	wild olive	*	-	-
35.	***L.vineacola* Sturhan and Weischer 1954**	AR113	Córdoba (Córdoba, Spain)	wild olive	*	-	-
36.	***L.vineacola* Sturhan and Weischer 1954**	JAO01	Villaviciosa (Córdoba, Spain)	culti. live	*	-	-
37.	***L. vinearum* Bravo and Roca 1995**	AR059	Santa Mª de Trassierra (Córdoba, Spain)	wild olive	KT308874	KT308892	KT308903

Species	Sampling site code	Administrative locality	Host-plant	D2-D3	ITS1	Partial 18S
38. ***L. vinearum* Bravo and Roca 1995**	AR066	Santa Mª de Trassierra (Córdoba, Spain)	wild olive	KT308875	KT308893	-
39. ***L. vinearum* Bravo and Roca 1995**	AR097	Santa Mª de Trassierra (Córdoba, Spain)	wild olive	KT308876	-	-
40. ***L. vinearum* Bravo and Roca 1995**	AR111	Santa Mª de Trassierra (Córdoba, Spain)	wild olive	KT308877	-	-

(-) Not obtained or not performed.
(*) Sequenced population but not deposited in GenBank database, since was identical to other sequences of the same species.

2.4 DNA extraction, PCR and sequencing

For molecular analyses, in order to avoid mistakes in the case of mixed populations in the same sample, two live nematodes from each sample were temporary mounted in a drop of 1M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed in form to the unidentified populations of *Longidorus*. Morphometrics and photomicrographs recorded during this initial study were not used as part of the morphological study or analyses. Following morphological confirmation, the specimens were removed from the slides and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (Castillo *et al.* 2003). One nematode specimen of each sample was transferred to an Eppendorf tube containing 16 µl ddH2O, 2 µl 10x PCR buffer and 2 µl proteinase K (600 µg/ml) (Promega, Benelux, The Netherlands) and crushed during 2 min with a micro-homogeniser, Vibro Mixer (Zürich, Switzerland). The tubes were incubated at 65°C (1 h), then at 95°C (15 min), and finally at 80°C (15 min). One µl of extracted DNA was transferred to an Eppendorf tube containing: 2.5 µl 10X NH4 reaction buffer, 0.75 µl MgCl2 (50mM), 0.25 µl dNTPs mixture (10mM each), 0.75 µl of each primer (10mM), 0.2 µl BIOTAQ DNA Polymerase (BIOLINE, UK) and ddH2O to a final volume of 25 µl. The D2–D3 expansion segments of 28S rRNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Nunn 1992). The ITS1 region was amplified using forward primer 18S (5´TTGATTACGTCCCTGCCCTTT-3´) (Vrain *et al.* 1992) and reverse primer rDNA1 (5´-ACGAGCCGAGTGATCCACCG-3´) (Cherry *et al.* 1997). Finally, the portion of the 18S-rRNA was amplified using primers 988F (5´-CTCAAAGATTAAGCCATGC-3´), 1912R (5´TTTACGGTCAGAACTAGGG-3´), 1813F (5´- CTGCGTGAGAGGTGAAAT-3´) and 2646R (5´-GCTACCTTGTTACGACTTTT-3´) (Holterman *et al.* 2006).

PCR cycle conditions were: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing temperature of 55°C for 45 s, 72°C for 3 min, and finally one cycle of 72°C for 10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred to above. The resulting products were purified

and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 6.1.

2.5 Phylogenetic analysis

D2–D3 expansion segments of 28S rRNA, ITS1, and partial 18S rRNA sequences of different *Longidorus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (He *et al.* 2005, Holterman *et al.* 2006, Palomares-Rius *et al.* 2008, Coomans *et al.* 2012, Gutiérrez-Gutiérrez *et al.* 2013). The newly obtained and published sequences for each gene were aligned using MAFFT ver. 7 (Katoh *et al.* 2002), strategy FFT-NS-1 with default parameters. Sequence alignments were manually edited using BioEdit (Hall 1999). Percentage similarity between sequences was calculated using the sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all gap or place-holding characters were treated as a gap (Hall 1999). When positions of both sequences have a gap they do not contribute (Hall 1999). Phylogenetic analyses of the sequence data sets were performed based on Bayesian inference (BI) using MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). The best fitted model of DNA evolution was obtained using JMODELTEST v. 2.1.7 (Darriba *et al.* 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analyses were performed under SYM+I+G (namely, symmetrical of invariable sites and a gamma-shaped distribution) model for D2–D3 expansion segments of 28S rRNA, TVM+I+G and TIM3+I+G (namely, transversional and a transitional of invariable sites and a gamma-shaped distribution) models for the two ITS1 region datasets, TVMef+I+G (namely, equal-frequency transversional of invariable sites and gamma-shaped distribution) model for the partial 18 S rDNA. These BI analyses were run

separately per dataset using four chains for 2 × 106, 1 and 1 × 106, and 3 × 106 generations, respectively. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996).

2.6 Nomenclatural Acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature (ICZN), and hence the new names contained herein are available under that Code from the electronic edition. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank Life Science Identifiers (LSIDs) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn: lsid:zoobank.org:pub: C8230A9DFD45-4AA4-9ABF-8445E8001CCC. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS.

3. Results

3.1 Taxon sampling, abundance and prevalence of *Longidorus* species

All positive *Longidorus* spp.-sampling sites for this study, including specimens used in morphological and/or genetic analyses, are shown in Table 6.1 and Figure 6.1. Ten *Longidorus* species were associated with wild olive (*viz. Longidorus alvegus* Roca, Pereira and Lamberti 1989 (Roca *et al.* 1989), *Longidorus indalus* sp. nov., *Longidorus intermedius* Kozlowska and Seinhorst 1979, *L. lusitanicus, Longidorus oleae* Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius and

Castillo 2013, *Longidorus silvestris* sp. nov., *Longidorus vallensis* sp. nov., *Longidorus vineacola* Sturhan and Weischer 1964, *L. vinearum*, and *Longidorus wicuolea* sp. nov.), whereas nine *Longidorus* species (*viz. L. alvegus*, *Longidorus indalus* sp. nov., *Longidorus macrodorus* sp. nov., *L. magnus*, *L. oleae*, *Longidorus onubensis* sp. nov., *Longidorus vallensis* sp. nov., *L. vineacola*, and *Longidorus wicuolea* sp. nov.) were associated with cultivated olive in Andalusia (Table 6.1, Figure 6.1). Except for *L. alvegus*, *L. indalus* sp. nov. and *L. vineacola*, that occurred in both olive types, all the remaining identified species where present only in either wild or cultivated olives.

Longidorus spp. were present in low to moderate densities (from 1 to 33 nematodes per 500 cm^3 of soil), and were moderately distributed in both wild and cultivated olives (Table 6.2). The overall prevalence of *Longidorus* spp. in wild olives was 16.03% (21 out of 131 samples) whereas in cultivated olives was 5.97% (19 out of 318 samples) (Tables 6.1 and 6.2). Although wild and cultivated olives were present in all of the eight provinces of Andalusia, the genus *Longidorus* was not detected in Jaén and Málaga provinces, and in Granada only in cultivated olives (4 samples out of 39) (Table 6.2, Figure 6.1). The three most prevalent *Longidorus* species, *L. indalus* sp. nov., *L. oleae*, and *L. vineacola*, were detected in both wild and cultivated olives, as well as *L. alvegus*, *L. vallensis* sp. nov. and *L. wicuolea* sp. nov. but with lower prevalence (Tables 6.1 and 6.2). *Longidorus vineacola* was rather moderately distributed among the studied zones having the highest overall prevalence in both wild and cultivated olives (Tables 6.1 and 6.2). However, some other *Longidorus* species showed a lower prevalence and were only detected either in wild (*L. lusitanicus*, *L. silvestris* sp. nov. and *L. vinearum*) or in cultivated olive (*L. macrodorus* sp. nov., *L. magnus*, *L. onubensis* sp. nov.) (Tables 6.1 and 6.2).

Table 6.2 Soil nematode population density (number of specimens) and prevalence (%) of *Longidorus* spp. in wild and cultivated olives in provinces of Andalusia, southern Spain.[a]

	Almería province		Cádiz province		Córdoba province		Granada province		Huelva province		Sevilla province	
***Longidorus* species**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**
number of samples	10	23	56	19	29	72	1	39	8	20	9	60
***L. alvegus* Roca *et al*., 1989**	-	-	-	-	-	-	-	-	3 (12.5)	-	1 (11.1)	12 (1.7)
***L. indalus* sp. nov.**	3 (20.0)	18 (21.7)	-	-	-	-	-	1 (5.1)	-	-	-	-
***L. intermedius* Kozlowska & Seinhorst, 1979**	-	-	33 (1.8)	-	-	-	-	-	-	-	-	-
***L. lusitanicus* Macara, 1986**	-	-	8 (1.8)	-	-	-	-	-	-	-	-	-
***L. macrodorus* sp. nov.**	-	-	-	-	-	1 (1.4)	-	-	-	-	-	-
***L. magnus* Lamberti *et al*., 1982**	-	-	-	-	-	3 (2.8)	-	3 (5.1)	-	-	-	-
***L. oleae* Gutiérrez-Gutiérrez et al., 2013**	-	-	-	-	2 (6.8)	-	-	-	-	-	2 (11.1)	2 (1.7)
***L. onubensis* sp. nov.**	-	-	-	-	-	-	-	-		3 (5.0)	-	-

	Almería province		Cádiz province		Córdoba province		Granada province		Huelva province		Sevilla province	
***Longidorus* species**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**	**Wild**	**cultivated**
***L. silvestris* sp. nov.**	-	-	11 (1.8)	-	-	-	-	-	-	-	-	-
***L. vallensis* sp. nov.**	-	-	3 (1.8)	-	-	3 (1.4)	-	-	-	-	-	-
***L.vineacola* Sturhan & Weischer, 1954**	-	-	4 (5.5)	1 (5.3)	1 (3.4)	1 (1.4)	-	-	1 (12.5)	-	-	4 (1.7)
***L. vinearum* Bravo & Roca, 1995**	-	-	-	-	14 (13.8)	-	-	-	-	-	-	-
***L. wicuolea* sp. nov.**	-	-	-	-	-	-	-	-	2 (12.5)	-	-	5 (1.7)

[a] Population density was calculated as the mean of *Longidorus* nematodes per 500 cm^3 of soil. The prevalence was computed by dividing the numbers of samples in which the *Longidorus* species was observed by the total number of samples and expressed as a percentage. Since no *Longidorus* spp. were detected in wild and cultivated olives in Jaén (9 wild olive and 58 cultivated olive samples) and Málaga (9 wild olive and 27 cultivated olive samples) provinces, data were not indicated in this table.
(-) not found

3.2 Taxonomic treatment

Nematoda Linnaeus, 1758
Dorylaimida Pearse, 1942
Longidoridae Thorne, 1935
Longidorinae Thorne, 1935
Longidorus Micoletzky 1992

3.2.1 *Longidorus indalus* sp. nov.

urn:lsid:zoobank.org:act:CE07DF59-E705-43D1-9CFF-1A8D793FA58D

Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (37°08'47.5"N, 002°43'31.7"W), at Las Tres Villas, Almería province, Spain; collected by G. Leon Ropero, April 11, 2013; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number ST41-21).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers ST41-01-ST41-17); two females at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (ST41-20); two females at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT837); and four females at USDA Nematode Collection, Beltsville, MD, USA (T-6629p); collected by G. Leon Ropero, April 11, 2013.

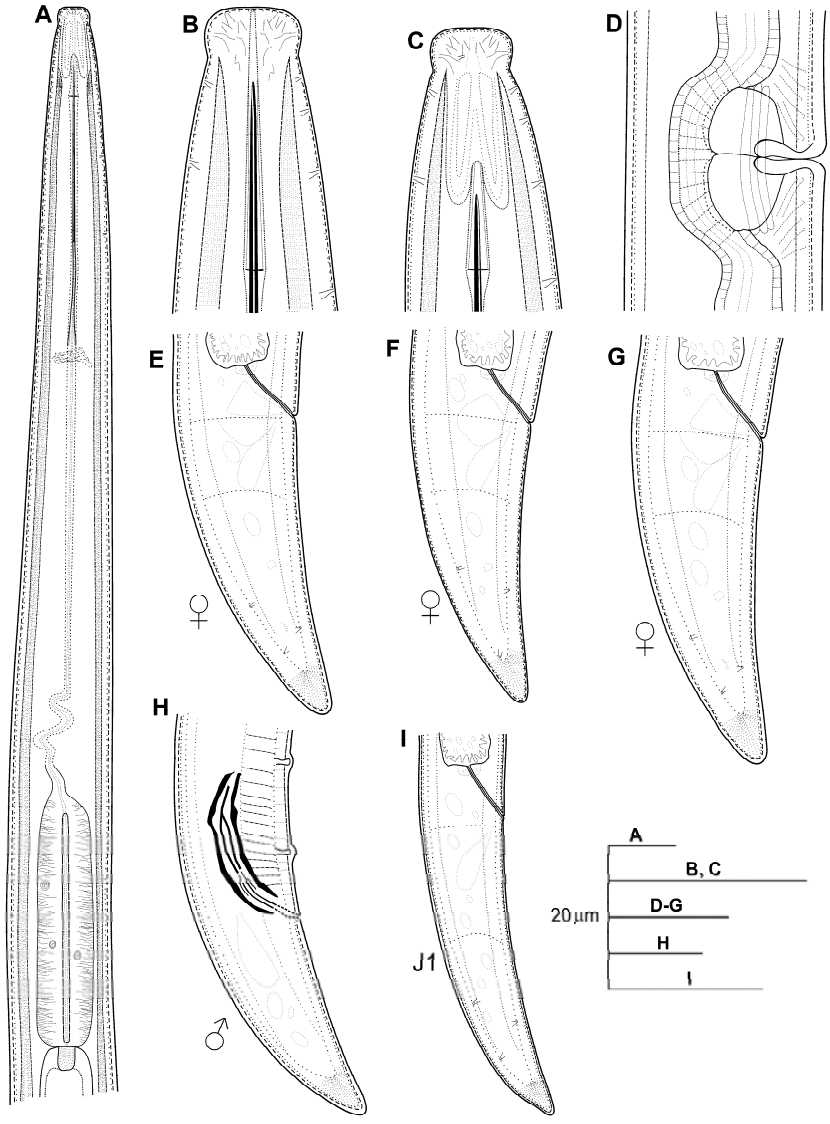

Figura 6.2: Line drawings of *Longidorus indalus* sp. nov. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E-G) Female tails. H) Male tail. I) First-stage juvenile tail (J1).

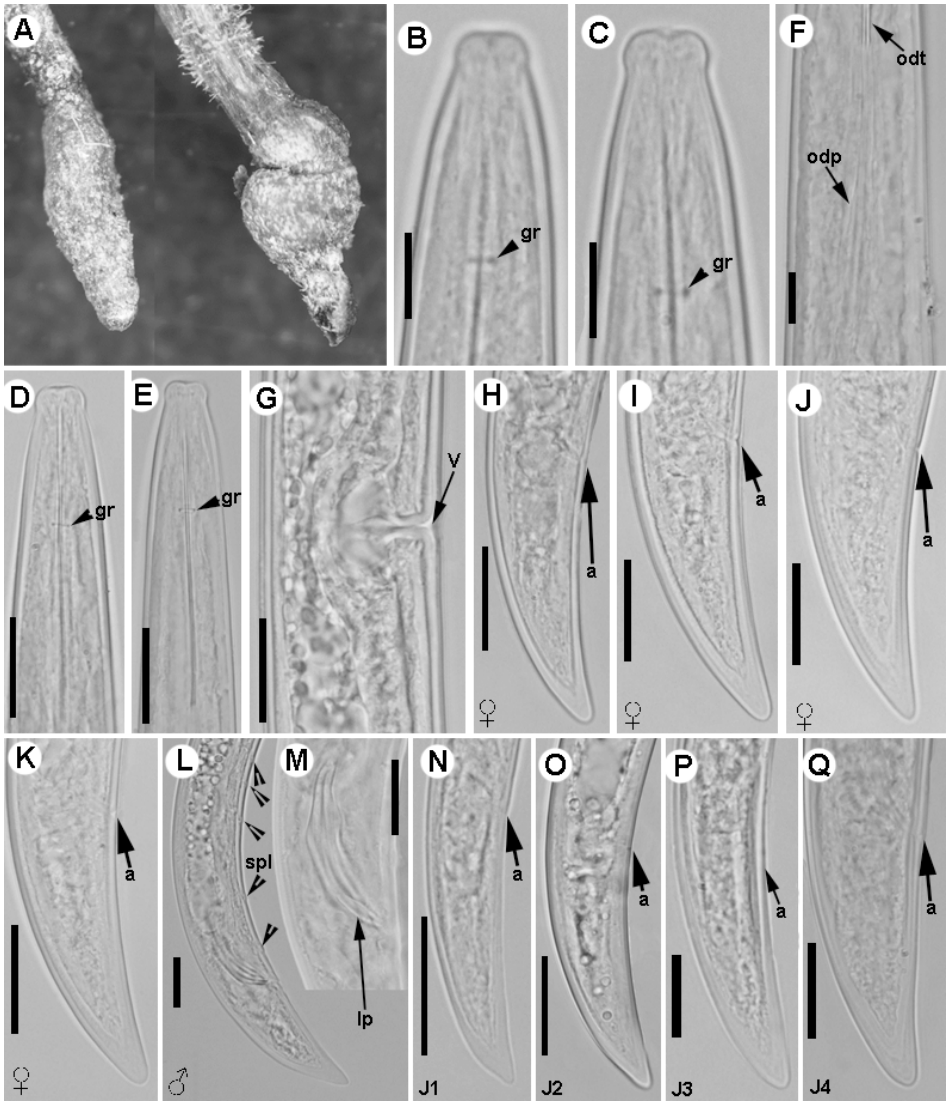

Figura 6.3: Light micrographs of *Longidorus indalus* sp. nov., female paratypes, male and juvenile stages. A) Olive apical galled roots infected by the nematode. B–E) Female anterior regions. F) Detail of odontostyle and odontophore. G) Vulval region. H-K) Female tails. L, M) Male tail with detail of spicules. N-Q) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; gr = guidingring; odt = odontostyle; odp = odontophore; lp = lateral accessory piece; spl = ventromedian supplements; v = vulva. Scale bars B, C, F = 10 µm; D, E, G-Q = 20 µm.

Diagnosis

Longidorus indalus sp. nov. is characterized by a moderate long body (4.1–6.0 mm), assuming an open C-shaped when heat relaxed; lip region expanded distinctly set off from body contour, 8.5–10.0 µm wide and 3.0–4.5 µm high; guiding-ring located 19.0–27.5 µm from anterior end; relatively short odontostyle (53.5–60.5 µm); amphidial fovea pocket-shaped, slightly asymmetrically bilobed; vulva almost equatorial; female tail long, conoid, and bearing three pairs of caudal pores; c' ratio (1.8–2.9); males extremely rare, only one male was found, with very short spicules (34.5 µm) and 5 ventromedian supplements; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KT308852-KT308854, KT308878-KT308879, and KT308894-KT308895, respectively). According to the polytomous key Chen et al. (Chen *et al.* 1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A1-B1-C2-D4-E2-F23-G3-H56-I12.

Etymology

The species name is derived from the name *'indalo'* a prehistoric symbol found in a cave of Almería, the province of the locality where the type specimens were collected.

Description of taxa

Female:

Body somewhat helicoid to arcuate, cylindrical, relatively long and thin, slightly tapering towards at both ends. When heat relaxed, body ventrally curved in open C-shaped. Cuticle thin appearing smooth under low magnifications, 1.9 ± 0.4 (1.5–2.5) µm thick at mid body, but slightly thicker (3.1 ± 0.8 (2.0–4.5) µm) and marked by very fine superficial transverse striate mainly in tail region, as shown by higher magnifications. Lip region expanded distinctly set off from body contour, anteriorly flattened, 9.2 ± 0.5 (8.5–10.0) µm wide and 3.9 ± 0.4 (3.0–4.5) µm high. Amphidial fovea pocket-shaped, slightly asymmetrically bilobed with lobes occupying about 1/3 part of distance between oral aperture and guiding-ring. Stylet guiding-ring single, located 2.8 ± 0.2 (2.5–3.2) times lip region diam. From anterior

end. Odontostyle typical of genus, 1.5 ± 0.2 (1.1–1.9) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak basal swellings. Nerve ring surrounding odontophore base at 94.3 ± 4.9 (85.5–107.0) µm from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb short and cylindrical, 92.3 ± 9.6 (72.0–103.5) µm long and 15.6 ± 1.8 (12.5–19.5) µm in diam. Glandularium 83.3 ± 8.7 (63.5–96.0) µm long. Dorsal pharyngeal gland nucleus (DN) and ventrosublateral nuclei (SVN) located at 33.5 ± 4.0 (27.3–39.5)% and 57.0 ± 4.4 (48.9–63.7)% of distance from anterior end of pharyngeal bulb, respectively. Nucleolus of DN larger than nucleoli of two SVN (4.0–4.5 vs 3.0–3.5 µm). Cardia conoid-rounded, 8.2 ± 0.2 (5.5–10.5) µm long. Lateral chord *ca* 9.6 µm wide at mid-body or *ca* 28% of corresponding body diam. Reproductive system with both genital branches equally developed, each branch 314–800 µm long, with reflexed ovaries very variable in length (85.5–161 µm long). Vulva in form of a transverse slit, located slightly anterior of the middle of the body, vagina perpendicular to body axis, 13.7 ± 3.2 (8.5–16.5) µm long or 24–47% of corresponding body width, surrounded by well-developed muscles. Genital branches equally developed, 9.7 ± 2.4 (6.8–13.9), 9.9 ± 2.2 (6.7–13.9)% of body length, respectively. Uteri highly variable in length (250–594 µm long), without sperm cells in all female specimens examined; sphincter well-developed, between uterus and oviduct. Eggs mature observed in some gravid female specimens along uterus from one gonoduct, 228.3 ± 8.0 (220.0–236.0) µm long and 32.2 ± 2.0 (30.0–34.0) µm wide. Anterior and posterior oviduct of similar size. Prerectum very variable in length, 673.1 ± 120.7 (489.0–861.0) µm long, and rectum 17.9 ± 3.2 (8.5–16.5) µm long ending in anus as a small rounded slit. Tail long, bluntly conoid, with rounded terminus, bearing three pairs of caudal pores.

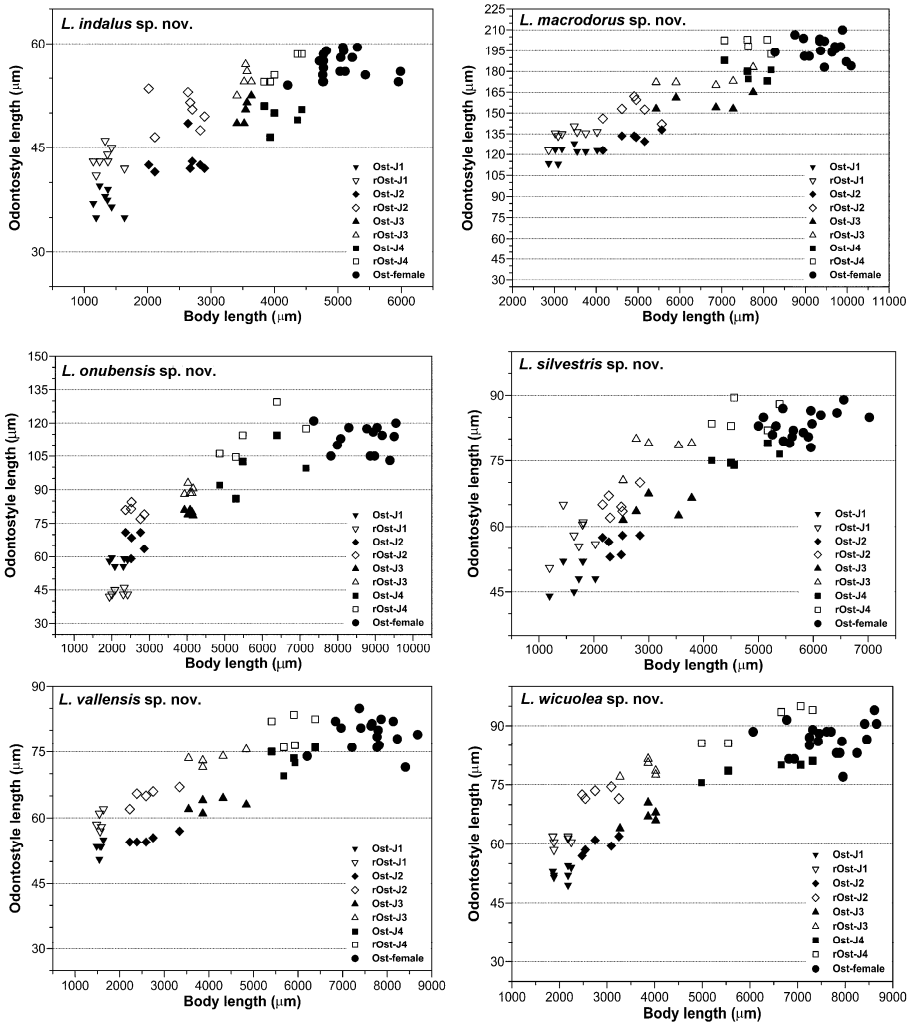

Figura 6.4: Relationship between body length and functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from first-stage juveniles (J1) to mature females of. A) *Longidorus indalus* sp. nov. B) *Longidorus macrodorus* sp. nov. C) *Longidorus onubensis* sp. nov. D*) Longidorus silvestris* sp. nov. E) *Longidorus vallensis* sp. nov. F) *Longidorus wicuolea* sp. nov.

Table 6.3 Morphometrics of females, males and juvenile stages of *Longidorus indalus* sp. nov. from the rhizosphere of cultivated and wild olives at several localities (Almería province) southern Spain[a].

Host/locality, sample code	**cultivated olive, Las Tres Villas (Almería province), ST041**						**Wild olive Agua Amarga (Almería province), AR46**
Characters/ratios[b]	**Holotype**	**Paratype Females**	**J1**	**J2**	**J3**	**J4**	**Females**
n		21	9	7	5	6	6
L (mm)	5.3	5.0 ± 0.42 (4.2-6.0)	1.34 ± 0.16 (1.14-1.64)	2.56 ± 0.35 (2.16-2.84)	3.54 ± 0.08 (3.41-3.64)	4.10 ± 0.24 (3.84-4.43)	4.6 ± 0.36 (4.1-5.1)
a	134.1	143.5 ± 14.4 (115.0-178.2)	78.2 ± 6.2 (71.5-89.5)	100.0 ± 14.2 (85.8-128.6)	124.4 ± 10.5 (115.4-140.9)	124.7 ± 9.1 (118.3-142.7)	129.7 ± 10.6 (116.9-145.6)
b	14.7	15.3 ± 2.5 (10.2-19.1)	7.7 ± 2.0 (5.1-11.3)	10.0 ± 0.9 (9.0-11.7)	12.0 ± 2.9 (9.7-16.9)	14.9 ± 2.5 (10.8-17.1)	18.7 ± 3.4 (15.6-24.2)
c	95.4	98.2 ± 10.4 (81.0-122.8)	33.2 ± 2.2 (30.9-37.6)	50.3 ± 6.6 (40.3-58.0)	66.2 ± 4.5 (62.2-73.6)	77.8 ± 7.2 (71.2-91.5)	103.8 ± 10.1 (89.1-113.7)
c´	2.5	2.3 ± 0.2 (1.9-2.9)	3.4 ± 0.2 (3.2-3.8)	3.0 ± 0.3 (2.5-3.4)	2.8 ± 0.2 (2.6-3.1)	2.4 ± 0.2 (2.0-2.6)	1.9 ± 0.1 (1.8-2.1)
V	48.5	47.6 ± 1.2 (45.5-50.0)	-	-	-	-	49.8 ± 1.8 (47.0-52.0)
Odontostyle	56.5	56.8 ± 1.8 (54.0-59.5)	37.2 ± 1.7 (35.0-39.5)	43.1 ± 2.4 (41.5-48.5)	50.3 ± 1.8 (48.5-52.5)	49.9 ± 2.0 (46.5-52.5)	55.2 ± 1.4 (53.5-57.5)
Host/locality, sample code	**cultivated olive, Las Tres Villas (Almería province), ST041**						**Wild olive Agua Amarga (Almería province), AR46**

Characters/ratios[b]	Holotype	Paratype Females	J1	J2	J3	J4	Females
Odontophore	35.0	37.5 ± 4.7 (30.0-51.0)	22.5 ± 1.6 (20.0-24.0)	26.9 ± 3.8 (21.5-30.5)	34.9 ± 2.0 (32.5-36.5)	30.2 ± 7.0 (25.0-42.5)	41.3 ± 3.7 (37.0-46.0)
Replacement odontostyle	-	-	43.4 ± 1.6 (41.0-46.0)	50.3 ± 2.6 (46.5-53.5)	54.9 ± 1.7 (52.5-57.0)	55.9 ± 2.1 (54.0-58.5)	-
Lip region diam.	8.5	9.2 ± 0.5 (8.5-10.0)	6.6 ± 0.4 (6.0-7.5)	7.6 ± 0.3 (7.0-8.0)	8.2 ± 0.8 (7.5-9.5)	8.6 ± 0.4 (8.0-9.0)	8.9 ± 0.4 (8.5-9.5)
Oral aperture-guiding ring	26.5	25.7 ± 1.1 (23.5-27.5)	16.6 ± 1.0 (15.5-18.0)	20.2 ± 1.3 (18.0-21.5)	22.4 ± 1.1 (21.5-24.0)	22.8 ± 0.9 (22.0-24.0)	22.9 ± 2.0 (19.0-24.0)
Tail length	55.5	51.7 ± 4.9 (45.5-59.5)	40.3 ± 2.6 (37.0-43.5)	50.7 ± 2.1 (47.5-54.0)	53.6 ± 3.7 (48.5-58.5)	53.8 ± 1.9 (51.0-56.5)	44.6 ± 3.7 (41.0-50.5)
J	11.5	10.0 ± 1.3 (7.5-12.0)	5.3 ± 0.7 (4.5-6.0)	6.2 ± 0.3 (6.0-6.5)	7.4 ± 1.2 (6.5-9.0)	7.8 ± 1.3 (6.5-9.0)	9.0 ± 0.8 (8.0-10.0)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Table 6.4 Morphometrics of females, males and juvenile stages of *Longidorus indalus* sp. nov. from the rhizosphere of cultivated and wild olives at several localities (Almería and Granada provinces) southern Spain[a].

Host-plant	**Cultivated olive**					**Wild olive**
Locality, sample code	**Tabernas (Almería province), JAO73**		**Las Tres Villas (Almería province), ST042**	**Lecrín (Granada province), ST193**	**Sorbas (Almería province), ST045**	**Sorbas (Almería province), AR044**
Characters/ratios[b]	**Females**	**Males**	**Females**	**Female**	**Females**	**Female**
n	3	1	3	1	3	1
L (mm)	4.7 ± 0.27 (4.5-5.0)	4.3	5.2 ± 0.42 (4.8-5.7)	4.3	5.0 ± 0.23 (4.8-5.3)	5.7
a	137.6 ± 17.0 (119.9-153.8)	155.4	136.2 ± 19.1 (119.2-156.9)	152.3	140.5 ± 14.5 (126.2-155.2)	128.2
b	15.7 ± 2.1 (13.6-17.7)	20.0	14.5 ± 0.4 (14.2-14.9)	18.6	14.9 ± 1.2 (14.1-16.3)	13.8
c	98.8 ± 7.3 (93.3-107.1)	97.1	104.9 ± 0.4 (98.6-112.6)	92.4	98.0 ± 1.6 (97.0-99.9)	109.7
c´	2.1 ± 0.3 (1.8-2.4)	1.9	2.1 ± 0.1 (2.0-2.3)	2.4	2.4 ± 0.2 (2.2-2.6)	2.0
V or T	48.3 ± 2.1 (46.0-50.0)	27.3	47.5 ± 0.5 (47.0-48.0)	48.0	46.5 ± 1.5 (45.0-48.0)	50.5
Odontostyle	58.8 ± 2.5 (56.0-60.5)	57.5	57.8 ± 1.1 (57.0-58.5)	60.0	55.8 ± 1.3 (54.5-57.0)	59.5
Host-plant	**Cultivated olive**					**Wild olive**

Locality, sample code	Tabernas (Almería province), JAO73		Las Tres Villas (Almería province), ST042	Lecrín (Granada province), ST193	Sorbas (Almería province), ST045	Sorbas (Almería province), AR044
Characters/ratios[b]	Females	Male	Females	Female	Females	Female
Odontophore	35.3 ± 4.0 (31.0-39.0)	39.0	37.0 ± 1.4 (36.0-38.0)	40.0	36.8 ± 2.9 (33.5-39.0)	41.5
Lip region diam.	8.8 ± 0.6 (8.5-9.5)	8.5	9.0 ± 0.7 (8.5-9.5)	9.5	8.8 ± 0.6 (8.5-9.5)	8.5
Oral aperture-guiding ring	24.8 ± 0.8 (24.0-25.5)	25.5	25.0 ± 0.0 (25.0-25.0)	24.0	25.0 ± 1.3 (23.5-26.0)	23.5
Tail length	47.8 ± 0.8 (47.0-48.5)	44.0	49.5 ± 4.8 (45.0-54.5)	47.0	51.3 ± 3.1 (48.0-54.0)	52.0
Spicules	-	34.5	-	-	-	-
Lateral accessory piece	-	13.5	-	-	-	-
Supplements	-	5	-	-	-	-
J	9.5 ± 0.5 (9.0-10.0)	9.5	10.3 ± 0.8 (9.5-11.0)	9.5	9.2 ± 1.2 (8.5-10.5)	10.0

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Table 6.5 Identity matrix, percentage (%) of identical residues between (indels included) rDNA sequences amongst *Longidorus* species. Above diagonal D2-D3 expansion segments of 28S rRNA and below diagonal internal transcribed spacer 1 (ITS1) region*.

	***Longidorus* spp.**																								
***Longidorus* spp.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
1. *L. indalus* sp. nov.*		85	87	88	**91**	87	89	83	85	88	-	87	87	88	-	87	87	88	88	86	84	89	**92**	**90**	87	87
2. *L. macrodorus* sp. nov.	-		86	88	87	88	86	85	**91**	86	-	87	84	87	-	83	**91**	87	87	88	86	83	88	83	**91**	87
3. *L. onubensis* sp. nov.	-	62		**91**	88	**91**	87	86	88	85	-	**94**	84	**95**	-	85	89	**93**	**94**	**90**	88	85	88	85	**92**	**95**
4 *L. silvestris* sp. nov.	-	46	50		89	**98**	88	87	88	87	-	**91**	86	**92**	-	86	**90**	**92**	**92**	**90**	88	84	89	86	**92**	**92**
5. *L. vallensis* sp. nov.	-	51	51	42		89	**91**	84	87	88	-	88	88	89	-	86	89	89	89	87	85	89	**96**	89	89	88
6. *L. wicuolea* sp. nov.	-	46	52	88	41		87	86	88	86	-	**91**	86	**91**	-	85	89	**91**	**92**	89	87	84	89	86	**91**	**92**
7. *L. alvegus*	-	46	47	46	39	48		84	86	90	-	87	86	87	-	86	87	88	88	87	85	87	91	88	88	87
8. *L. andalusicus*	-	-	-	-	-	-	-		84	83	-	86	85	87	-	87	87	86	87	89	**94**	84	84	82	87	86
9. *L. baeticus*	-	69	62	46	49	47	44	-		85	-	88	84	89	-	83	89	88	89	88	86	83	87	83	89	88
10. *L. breviannulatus*	53	-	-	-	-	-	-	-	-		-	85	85	86	-	87	88	86	86	86	84	87	**90**	88	87	86
11. *L. crassus*	57	-	-	-	-	-	-	-	-	52		-	-	-	-	-	-	-	-	-	-	-	**-**	-	-	-
12. *L. crataegi*	-	-	-	-	-	-	-	-	-	-	-		84	**96**	-	84	89	**94**	**95**	89	87	84	88	85	**92**	**95**

	Longidorus spp																								
***Longidorus* spp.**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**
2713. *L. elongatus*	-	56	55	40	55	40	43	-	53	-	-	-		84	-	**95**	85	85	84	85	87	88	89	87	86	85
14. *L. goodeyi*	-	-	-	-	-	-	-	-	-	-	-	-	-		-	85	89	**95**	**96**	**90**	89	85	89	86	**93**	**96**
15. *L. grandis*	57	-	-	-	-	-	-	-	-	52	84	-	-	-		-	-	-	-	-	-	-	-	-	-	-
16. *L. intermedius*	-	55	55	43	53	43	44	-	55	-	-	-	-	-	-		84	84	84	85	87	87	88	86	86	84
17. *L. iuglandis*	-	69	63	46	49	45	45	-	65	-	-	-	-	-	-	55		**90**	**90**	89	88	85	88	86	**91**	**90**
18. *L. lusitanicus*	-	63	79	50	50	50	46	-	62	-	-	-	-	-	-	55	62		**95**	89	87	85	89	85	**92**	**94**
19. *L. magnus*	-	64	85	50	51	51	48	-	64	-	-	-	-	-	-	56	65	80		89	88	85	89	85	**93**	**97**
20. *L. oleae*	-	60	58	42	47	43	41	-	58	-	-	-	-	-	-	52	58	57	60		**91**	85	88	84	**91**	89
21. *L. orientalis*	-	66	62	44	48	45	42	-	62	-	-	-	-	-	-	53	68	61	63	64		87	86	84	**90**	88
22. *L. profundorum*	51	-	-	-	-	-	-	-	-	53	53	-	-	-	53	-	-	-	-	-	-		**90**	88	86	85
23. *L. rubi*	-	51	50	38	66	37	39	-	51	-	-	-	-	-	-	48	49	49	50	46	48	-		**90**	89	88
24. *L. sturhani*	56		-	-	-	-	-	-	-	57	60	-	-	-	60	-	-	-	-	-	-	58	-		87	85
25. *L. vineacola*	-	63	69	51	51	51	49	-	62	-	-	-	-	-	-	54	65	69	70	58	64	-	50	-		**92**
26. *L. vinearum*		64	84	51	51	52	47		64	-	-	-	-	-	-	55	64	78	**91**	58	62	-	50	-	70	

* Similarity between sequences ≥ 90% are in bold letters. (-) Sequences not available or comparison not carried out because of low homology between sequences.

Male:

Extremely rare, only one male specimen was found but not in type locality. Morphologically similar to female except for genital system, but with posterior region slightly curved ventrally. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Tail conoid, dorsally conoid and ventrally concave with rounded terminus and thickened outer cuticular layer. Spicules very short, moderately developed and slightly curved ventrally; lateral guiding pieces more or less straight or with curved proximal end. Low number of supplements, one pair of adanal and 4 mid-ventral supplements.

Description of juveniles:

Morphologically similar to adults, but smaller. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 6.3, Figures 6.3 and 6.4; Robbins *et al.* 1995, 1996). J1s were characterised by a bluntly conoid tail with a c' ratio ≥ 3.2, well curved dorsally with a dorsal depression at hyaline region level (Figure 6.3) odontostyle length *ca* 37 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages. However, morphology in second-, third- and fourth-stages (except for undeveloped genital structures) similar to that of female, including conoid tail shape, becoming progressively shorter and stouter in each moult and shorter distance from anterior end to guiding-ring in each moult.

Measurements, morphology and distribution:

Morphometric variability is described in Tables 6.3 and 6.4 and morphological traits in Figures 6.2-6.4. In addition to the type locality, *Longidorus indalus* sp. nov. was extracted from five cultivated olive samples causing enlarged swellings of root tips (Figure 6.3, and two wild olive samples of several localities distributed in Almería and Granada province, being one of the two species (together with *L. magnus*) located on Eastern Andalusia (Table 6.1, Figure 6.1).

Relationships:

According to the polytomous key by Chen *et al*. (1997) and the supplement by Loof and Chen (1995), and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (lip region shape), and E (shape of amphidial pouch), *L. indalus* sp. nov. is closely related to *L. carpetanensis* and *L. unedoi* from which it can be differentiated by a combination of these characters discussed below, but particularly in female and male tail shape (bluntly conoid vs conical, dorsally convex) (Figures 6.3). *Longidorus indalus* sp. nov. differs from *L. carpetanensis* by a longer body length (4.1–5.7 vs 3.5–4.4 mm), higher a ratio (115.0–178.2 vs 96.0–118.0), slightly higher c and c´ ratio (81.0–122.8 vs 77.0–96.0, 1.8–2.9 vs 1.6–2.2, respectively), and a lower frequency of males (extremely rare vs frequent) (Arias *et al.* 1986). On the other hand, *L. indalus* sp. nov. differs from *L.unedoi* in shaving lower c and V ratio (81.0–122.8 vs 122.0–156.0, 42.0–52.0 vs 52.0–58.0; respectively), and slightly higher c´ ratio (1.4–2.0 vs 1.8–2.9) (Arias *et al.* 1986). Finally, *L. indalus* sp. nov. is molecularly related to *L. rubi* (Romanenko 1998) from which it can be mainly differentiated morphologically in having a smaller odontostyle and spicules length (53.5–60.5 vs 72.0–90.0, 35.0 vs 40.0–45.0 µm; respectively), and lower number of ventromedian supplements in male tail (5 vs 11–12) (Romanenko 1998, Gutiérrez-Gutiérrez *et al.* 2013).

Molecular divergence of the new species:

D2–D3 region of *L. indalus* sp. nov. (KT308852-KT308854) was 91% similar to several *Longidorus* species such as *L. closelongatus* (KJ808866), *L. pseudoelongatus* (KJ802873) and *L. rubi* (JX4455116) (Table 6.5). *Longidorus indalus* sp. nov. showed a high homogeneity for the D2–D3 region (99% similarity, 3 nucleotides) in the eight sampled populations. However, this homogeneity was lower for the ITS1 sequences (KT308878-KT308879) (98% similar, 23 nucleotides and 17 gaps). Some di- and tri-nucleotides microsatellites, (TA)n and (TGG)n, were found in the population from Lecrín, Granada province (KT308854) contributing to sequence variation. Low homologies in the GenBank were found for ITS1 sequence, the closest species in relation to this marker were *L. crassus* (AF511414) and *L. grandis* (AF511419), with a similarity of 70% only. The partial 18S of *L. indalus* sp. nov. (KT308894-KT308895) closely matched (99% similarity)

those for *L. closelongatus* (KJ802897), *L. crassus* (AY283158) and *L. grandis* (AY283165).

3.2.2 *Longidorus macrodorus* sp. nov.

urn:lsid:zoobank.org:act:9A8C0479-3145-4781-B749-027654C7B8E2

Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (38°22'33.9"N, 005°20'46.9"W), at La Grajuela, Córdoba province, Spain; collected by J. Martin Barbarroja, February 19, 2015; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number JAO6-01).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers JAO6-05-JAO6-20); one female and one male at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (JAO6-02); one female and one male at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT839); and one female and one male at USDA Nematode Collection, Beltsville, MD, USA (T-6630p); collected by J. Martin Barbarroja, February 19, 2015.

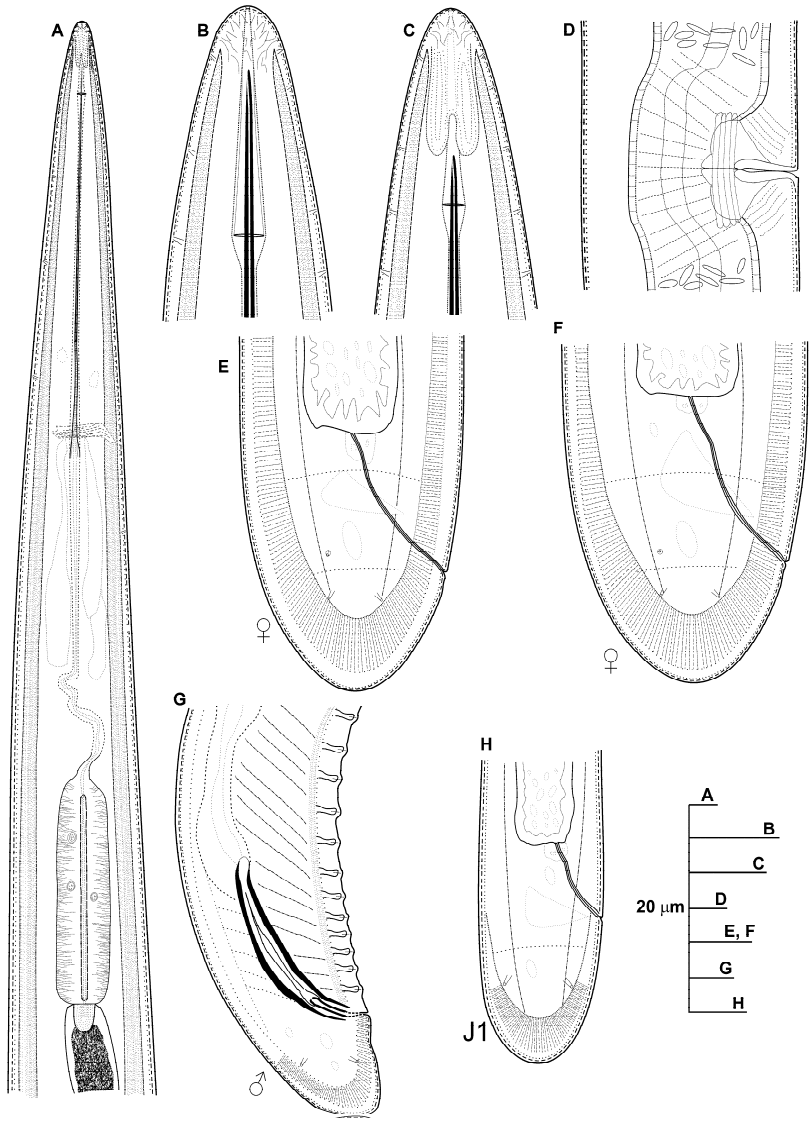

Figura 6.5: Line drawings of *Longidorus macrodorus* sp. nov., female paratypes, male and first-stage juvenile. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E-F) Female tails. G) Male tail. H) First-stage juvenile tail (J1).

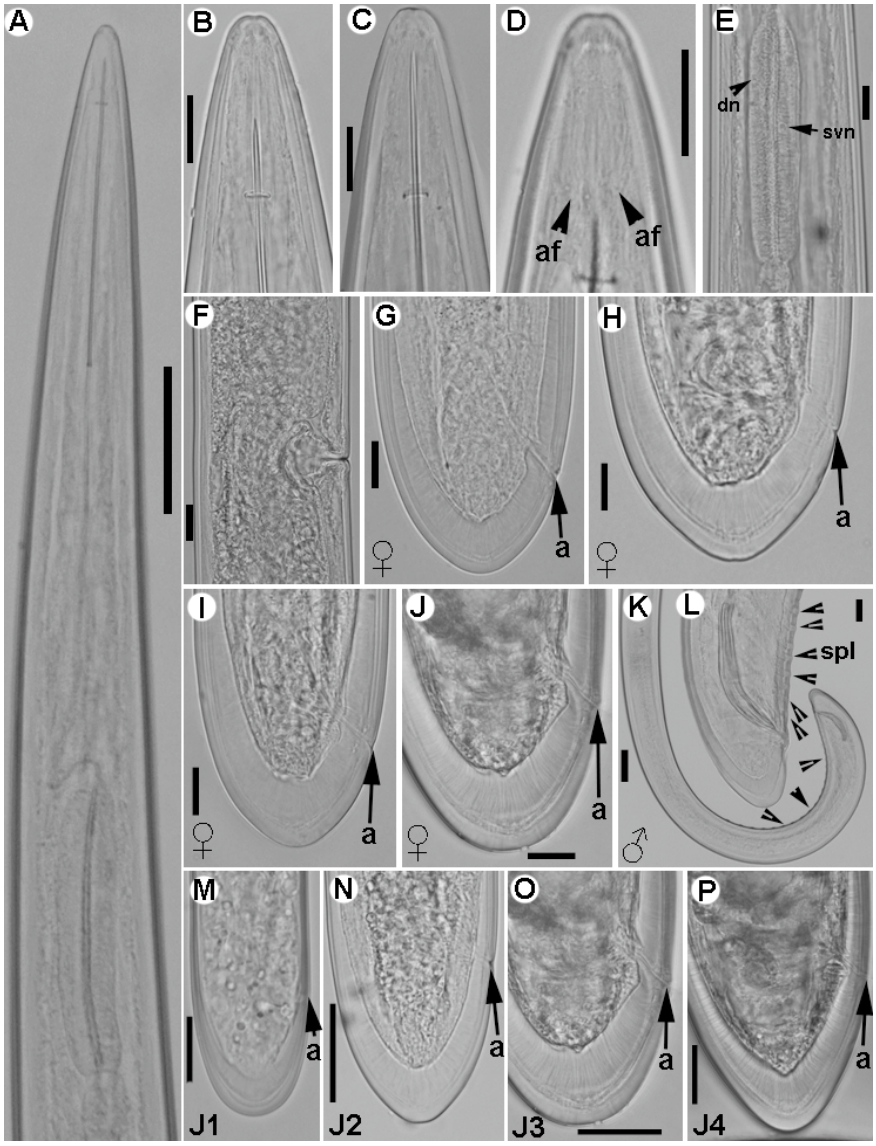

Figura 6.6: Light micrographs of *Longidorus macrodorus* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B–D) Female anterior regions. E) Detail of basal bulb. F) Vulval region. H-J) Female tails. K, L) Male tail with detail of spicules. M-P) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; af = amphidial fovea; dn = dorsal nucleus; spl = ventromedian supplements; svn = subventral nucleus. Scale bars A, K = 100 µm; B-J, L-P = 20 µm.

Diagnosis

Longidorus macrodorus sp. nov. is a gonochoristic species characterized by a very long body (9.3–10.1 mm), assuming a straight to nearly straight body when heat relaxed; lip region conoid-narrowed continuous with body contour, 8.5–12.0 µm wide; guiding-ring located 45.5–55.0 µm from anterior end; very long odontostyle (183.0–210.0 µm); amphidial fovea pocket-shaped, symmetrically bilobed; vulva almost equatorial; female tail short, bluntly conoid, and bearing between three and four pairs of caudal pores; c' ratio (0.5–1.0); males as frequently as females with long spicules (90.0–112.0 µm) and 17–25 ventromedian supplements; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KT308855-KT308856, KT308880-KT308881, and KT308896, respectively). According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), the new species has the following code: A7-B1-C45-D1-E2-F54-G12-H1-I2.

Etymology

The species name refers to the primarily distinguishing character of the long odontostyle (from Greek *macros* = long, and *dorus* = stylet).

Description of taxa

Female:

Body very long and rather robust, sharply tapering towards anterior end, usually assuming a body straight or nearly so shape when heat relaxed. Cuticle very finely striated generally but mainly at the posterior extremity, 5.5 ± 0.7 (4.0–7.0) µm thick at mid-body but more thickened at tail tip where it is 13.5 ± 3.0 (8.5–17.0) µm thick, immediately anterior to anus. Lip region conoid-narrowed, anteriorly rounded, and continuous with body contour. Amphidial fovea pocket-shaped slightly symmetrically bilobed with lobes about equal length and extending about 2/3 part of distance between oral aperture and guiding-ring, openings obscure appearing as minute pores, not slit-like. Stylet guiding-ring single, located 5.3 ± 0.6 (4.1–6.0) times lip region diam. from anterior end. Lateral chord 26.2 ± (24.0–30.0) µm wide at mid-body or 20–27% of corresponding body diam. Odontostyle very long and robust straight or slightly arcuate, 3.9 ± 0.3 (3.4–4.0) times as long as distance between anterior end to guiding-ring, odontophore about 2/3 part

of the odontostyle length, weakly developed with slightly enlarged at the base. Nerve ring encircling cylindrical part of pharynx at odontophore base, located 271.5 ± 10.3 (252.5–288.0) µm from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb long and cylindrical, 182.2 ± 9.3 (166.0–197.0) µm long or ca one-fourth of neck length, and 36.5 ± 3.7 (28.0–45.0) µm in diam. Glandularium 156.9 ± 8.8 (144.5–172.0) µm long. Normal arrangement of pharyngeal glands (Chen *et al.* 1997, Loof and Chen 1999): nuclei of the dorsal (DN) and subventral (SVN) glands situated at 26.2 ± 4.0 (21.0–33.0)% and 51.1 ± 3.1 (45.7–55.0)% of the distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nucleus (DN) slightly larger than nuclei of two SVN (4.0–6.0 vs 3.5–5.0 µm in diam.). Cardia hemispherical, 18.7 ± 3.9 (14.5–25.0) µm long. Reproductive system with both genital branches equally developed, relatively short compared to body length, ranging between 622–1318 µm long, with reflexed ovaries very variable in length. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, extending to *ca* 2/3 corresponding body width, surrounded by well-developed muscles. Genital branches equally developed, 8.8 ± 1.6 (6.6–13.0), 8.8 ± 2.0 (6.5–14.0)% of body length, respectively. Uterus short, thick-walled, filled with sperm cells in most female specimens observed; well-developed sphincter between uterus and *pars dilatata oviductus*, usually containing numerous sperm cells too. Ovaries equally developed and very variable in length, 192–545 µm long, both of them with a single row of oocytes. Prerectum variable in length, 2170 ± 559.7 (1427–3045) µm long, and rectum 46.6 ± 7.9 (36.0–56.0) µm long, anus a small rounded slit. Tail short, bluntly conoid, rounded to almost hemispherical, bearing between three and four pairs of caudal pores.

Table 6.6 Morphometrics of females, males and juvenile stages of *Longidorus macrodorus* sp. nov. from the rhizosphere of cultivated olive at La Grajuela (Córdoba province) southern Spain[a].

		Paratype		juvenile-stages			
Characters/ratios[b]	Holotype	Females	Males	J1	J2	J3	J4
n		19	20	8	6	5	5
L (mm)	9.3	9.3 ± 0.50 (8.3-10.1)	9.5 ± 0.77 (8.2-10.6)	3.37 ± 0.40 (2.86-4.02)	4.76 ± 0.39 (4.16-5.16)	6.65 ± 0.96 (5.43-7.75)	7.71 ± 0.45 (70.7-8.18)
a	92.0	83.1 ± 5.6 (73.6-92.0)	86.5 ± 9.6 (66.7-101.5)	85.4 ± 4.4 (76.4-89.4	86.0 ± 5.5 (81.2-94.4)	78.3 ± 1.6 (76.4-80.4)	82.2 ± 5.9 (75.6-90.9)
b	10.8	13.0 ± 1.5 (10.8-17.0)	13.4 ± 2.4 (9.4-19.0)	8.5 ± 1.5 (6.4-10.5)	10.0 ± 1.0 (8.7-11.0)	10.5 ± 2.3 (7.6-13.4)	12.1 ± 0.7 (11.1-12.8)
c	186.8	224.2 ± 40.2 (169.9-323.0)	200.4 ± 21.7 (155.9-246.0)	69.5 ± 17.3 (52.2-96.6)	89.7 ± 4.0 (85.4-94.6)	128.5 ± 26.4 (104.8-172.4)	167.2 ± 11.6 (157.1-184.0)
c´	0.7	0.7 ± 0.1 (0.5-1.0)	0.8 ± 0.1 (0.7-1.0)	1.6 ± 0.3 (1.2-2.0)	1.2 ± 0.1 (1.1-1.3)	1.0 ± 0.1 (0.8-1.1)	0.8 ± 0.1 (0.7-0.8)
V or T	46.0	48.9 ± 1.8 (46.0-52.0)	33.3 ± 5.4 (24.3-41.6)	-	-	-	-
Odontostyle	202.0	196.4 ± 7.7 (183.0-210.0)	197.2 ± 10.0 (181.5-220.0)	121.1 ± 5.2 (113.0-128.0)	130.4 ± 4.4 (123.0-133.5)	157.2 ± 5.5 (153.0-165.0)	179.3 ± 6.0 (173.0-188.0)
Odontophore	85.5	75.1 ± 8.7 (60.0-87.0)	73.1 ± 6.4 (59.0-86.5)	55.3 ± 7.3 (46.0-63.5)	51.4 ± 3.7 (48.0-57.5)	62.0 ± 7.4 (54.0-68.5)	66.7 ± 2.4 (63.0-69.5)

Characters/ratios[b]	Holotype	Paratype		juvenile-stages			
		Females	Males	J1	J2	J3	J4
Replacement odontostyle	-	-	-	134.5 ± 5.1 (123.0-140.5)	154.6 ± 6.3 (146.0-162.0)	174.0 ± 5.1 (170.0-183.0)	199.8 ± 4.6 (192.5-203.0)
Lip region diam.	8.5	9.7 ± 1.1 (8.5-12.0)	9.7 ± 1.1 (8.5-12.0)	6.0 ± 0.6 (5.0-6.5)	6.4 ± 0.4 (5.5-7.0)	8.1 ± 1.2 (7.0-10.0)	8.9 ± 1.2 (7.0-10.0)
Oral aperture-guiding ring	49.0	50.7 ± 2.8 (45.5-55.0)	51.2 ± 3.4 (45.0-60.0)	32.6 ± 1.9 (30.0-35.5)	37.3 ± 2.0 (34.0-39.5)	43.4 ± 2.7 (39.5-46.5)	47.8 ± 2.8 (45.0-50.5)
Tail length	50.0	42.9 ± 6.6 (30.0-54.0)	47.7 ± 4.4 (40.0-55.5)	50.4 ± 9.9 (36.0-64.0)	53.0 ± 2.5 (48.5-54.5)	53.8 ± 14.1 (31.5-66.0)	46.3 ± 3.8 (41.5-52.0)
Spicules	-	-	103.0 ± 5.3 (90.0-112.0)	-	-	-	
Lateral accessory piece	-	-	25.7 ± 2.3 (22.5-29.5)	-	-	-	-
J	25.0	23.7 ± 2.3 (20.0-28.0)	18.4 ± 1.4 (16.0-22.0)	12.4 ± 1.5 (9.5-13.5)	14.4 ± 1.1 (12.5-15.0)	19.3 ± 0.9 (18.5-20.0)	19.2 ± 2.2 (17.5-23.0)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Male:

Common, as frequently as female. Morphologically similar to female except for genital system, posterior region being more strongly coiled with slightly longer tail. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Spicules massive, robust, and curved ventrally; lateral guiding pieces more or less straight or with curved proximal end. Tail convex-conoid, dorsally conoid, ventrally being almost straight with broad blunt terminus and thickened outer cuticular layer. One pair of adanal supplements and 17–25 mid-ventral supplements.

Description of juveniles:

Morphometrics obtained from juvenile specimens, and of the relative lengths of body, tail, and functional and replacement odontostyle, confirmed the presence of four juvenile stages (Table 6.6, Figures 6.4 and 6.6; Robbins *et al.* 1995, 1996). J1s were characterised by a bluntly rounded to cylindrical tail with a c' ratio ≥ 1.2 (Table 6.6), an odontostyle very long, *ca* 120 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages.

Measurements, morphology and distribution:

Morphometric variability is described in Table 6.6, and morphological traits in Figures 6.4-6.7. *Longidorus macrodorus* sp. nov. was only found in the type locality in the rhizosphere of cultivated olive (Table 6.1, Figure 6.1).

Relationships:

L. macrodorus sp. nov. can be differentiated from all known species of the genus by a combination of characters, but particularly by its stylet and odontostyle length (252–288, 183–210 µm, respectively), the longest in the genus. Nonetheless, according to this morphometric character, included on matrix code A (odontostyle length) (Chen *et al.* 1997, Loof and Chen 1999), *L. macrodorus* sp. nov. groups with *L. ishigakiensis* Hirata 2002 and *L. tarjani* Siddiqi 1962. From *L. ishigakiensis* it differs mainly in having a longer body and odontostyle length (8.3–10.1 vs 5.3–6.9 mm, 183–210 vs

158–181 µm; respectively), lower a and c´ ratios (73.6–92.0 vs 106.0–130.0, 0.5–1.0 vs 1.0–1.2; respectively), higher c ratio (169.9–323.0 vs 133.0–169.0), amphidial pouch shape (symmetrically bilobed vs not bilobed, matrix code E2 vs E1), and presence vs absence of males. From *L. tarjani* the new species differs mainly by having a longer body and odontostyle length (8.3–10.1 vs 6.0–6.8 mm, 183–210 vs 178–182 µm; respectively), higher c ratio (169.9–323 vs 113–130), and lip region shape (rounded continuous vs set off from body contour, matrix code D1 vs D2). In addition, *L. macrodorus* sp. nov. is molecularly related to *L. baeticus* Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius and Castillo 2013 from which it can be mainly differentiated by a slightly longer body length (8.3–10.1 vs 6.5–9.4 µm), a longer odontostyle length (183.0–210.0 vs 111.0–133.0 µm), and slightly higher c ratio (169.9–323.0 vs 180.0–286.2) (Gutiérrez-Gutiérrez *et al.* 2013).

Molecular divergence of the new species:

The sequence divergences between *L. macrodorus* sp. nov. and other congeneric species were significant, D2–D3 sequences (KT308855-KT308856) were 91% similar to *L. baeticus* (JX445106-JX445107), *L. iuglandis* (JX445105) and *L. fasciatus* (JX445108) (Table 6.5). No intraspecific variation for the D2–D3 segments was detected between the two studied samples. ITS1 sequences (KT308880-KT308881) region also agree with results obtained from D2–D3, these sequences were 75% similar to *L. baeticus* (JX445093), *L. fasciatus* (JX445097) and *L. iuglandis* (JX445099). Similarity values for the partial 18S of *L. macrodorus* sp. nov. sequence (KT308896) with those deposited in GenBank were high and matched closely with several sequences such, as *L.vineacola* (AY283169) and *L. elongatus* (EU503141).

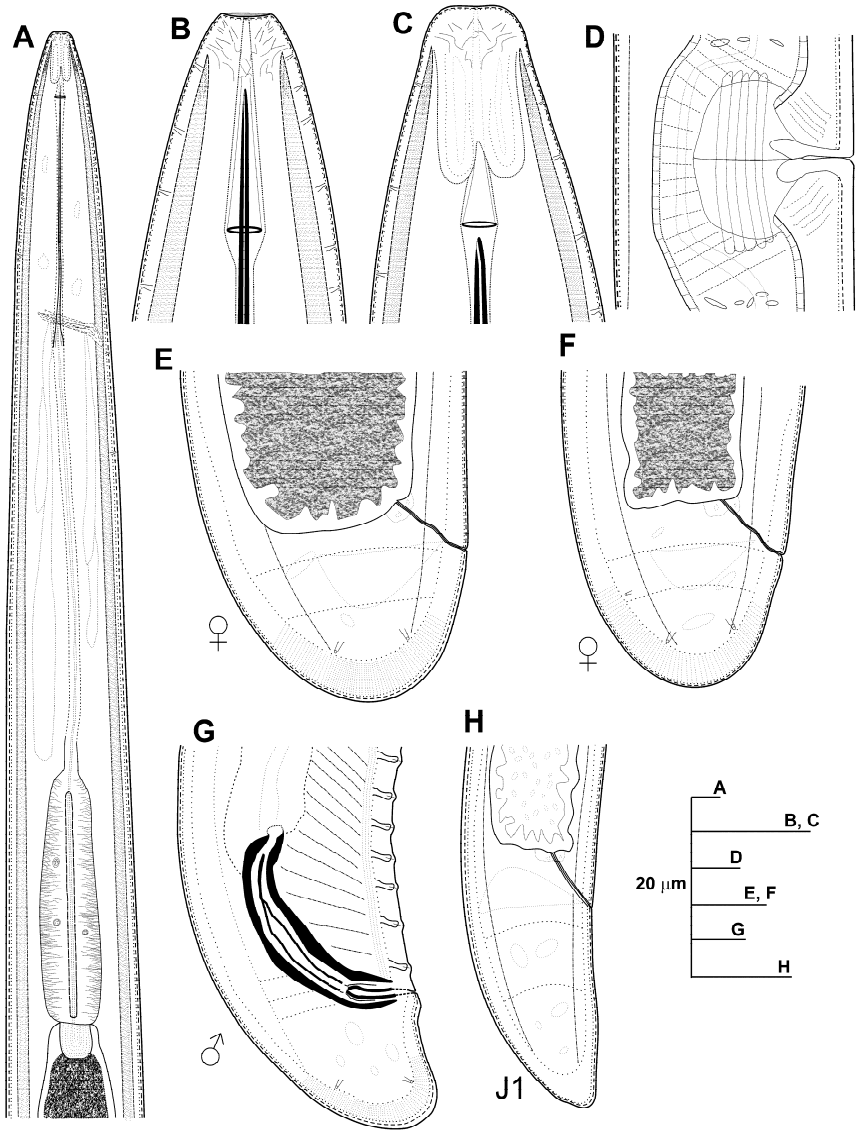

Figura 6.7: Line drawings of *Longidorus onubensis* sp. nov., female paratypes, male and first-stage juvenile. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E, F) Female tail. G) Male tail. H) First-stage juvenile tail (J1).

3.2.3 *Longidorus onubensis* sp. nov.

urn:lsid:zoobank.org:act:A9BE98FF-58A2-4BA4-8DFF-309D31C7D64F

Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (37°21'49.3"N, 006°39'56.8"W), Niebla, Huelva province, Spain; collected by J. Martin Barbarroja, January 21, 2012; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number ST5-13).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers ST5-02-ST5-12); one female and one male at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT842); and one female and male at USDA Nematode Collection, Beltsville, MD, USA (T-6631p); collected by J. Martin Barbarroja, January 21, 2012.

Diagnosis

Longidorus onubensis sp. nov. is a gonochoristic species characterized by a long and rather body (7.4–9.5mm), assuming an open C-shaped when heat relaxed; lip region broadly rounded to truncate, continuous or separated from body contour by slight depression, 14.0–16.5 µm wide; guiding-ring located 31–44µm from anterior end; long odontostyle (103–121 µm); amphidial fovea pocket-shaped with lobes of about equal length; vulva almost equatorial; female tail very short, broadly conoid to hemispherical, and bearing two or three pairs of caudal pores; c' ratio (0.6–0.8); males frequent with long spicules (92–98 µm) and 14–16 ventromedian

supplements; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KT308857-KT308858, KT308882-KT308883, and KT308897, respectively). According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A4-B2(3)-C34-D23-E2-F45-G2-H1-I2.

Etymology

The species epithet refers to *'Onuba'*, the Roman name of the province of Huelva, where the type specimens were collected.

Description of taxa

Female:

Body long and rather robust, slightly tapering towards anterior end, usually assuming an open C-shaped when heat relaxed, almost straight anteriorly and more curved behind the vulva to single spirals. Cuticle appearing smooth, 4.5 ± 0.8 (3.5–6.0) µm thick, 11.1 ± 2.3 (8.5–13.5) µm thick at tail tip, and marked by very fine superficial transverse striate mainly in tail region. Lip region broadly rounded frontally and more so laterally, separated from body contour by slight depression. However, lip region truncate, slightly concave anteriorly and continuous with body contour shape, observed in some female specimens. Amphidial fovea pocket-shaped symmetrically bilobed, with lobes of about equal length, occupying more of 2/3 part of distance between oral aperture and guiding-ring. Labial papillae prominent. Stylet guiding-ring single, located 2.5 ± 0.3 (2.1–3.0) times lip region diam. From anterior end. Lateral chord *ca* 25 µm wide at mid-body or one-fourth of corresponding body diam. Odontostyle moderate long and robust, usually straight, 1.9 ± 0.1 (1.6–2.29) times as long as odontophore; odontophore weakly developed, posterior slightly enlarged with rather weak basal swellings. Nerve ring encircling cylindrical part of pharynx, 11.3 ± 0.6 (10.2–12.3) times body width at lip region far from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb long and cylindrical, 149.7 ± 11.2 (135.0–173.0) µm long or *ca* one-third of neck length, 32.0 ± 3.7 (27.0–38.5) µm diam. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral pair of nuclei (SN)

situated slightly posterior to normal arrangement of pharyngeal glands (Chen *et al.* 1997, Loof and Chen 1999), 34.8 ± 4.2 (30.3–39.5)%, and 56.7 ± 7.1 (52.0–69.0)% of distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nucleus (DN) slightly larger than nuclei of two SVN (4.0–4.5 vs 3.5–4.0 µm in diam.). Glandularium 129.6 ± 11.8 (115.0–153.0) µm long. Cardia conoid-rounded, 12.3 ± 1.0 (11.5–13.5) µm long. Reproductive system with both genital branches equally developed, ranging between 456–989 µm long, with reflexed ovaries variable in length. *Pars dilatata oviductus* and uterus of about equal length, separated by a very strong and muscularised sphincter, on the external wall of which very cell body protrusions are present. Genital branches about equally developed, 7.4 ± 1.2 (6.1–9.4), 8.2 ± 1.5 (5.8–10.4)% of body length, respectively. Uterus wide and thick-walled, filled with little sperm cells in most female specimens observed. Ovaries equally developed 147–233 µm long, both of them with a single row of oocytes. Vulva in form of a transverse slit, approximately equatorial; vagina perpendicular to body axis, 42.0 ± 6.7 (30.0–50.5) µm long, or *ca* 42% of corresponding body width, surrounded by well-developed muscles. Prerectum very variable in length, 887.0 ± 331.1 (467.0–1155.0) µm long, and rectum 39.6 ± 4.4 (34.0–43.5) µm long, anus a small rounded slit. Tail very short, broadly conoid to hemispherical, with rounded terminus, bearing two or three pairs of caudal pores.

Male:

Common, but less frequent (40%) than female. Morphologically similar to female except for genital system, but with posterior region slightly curved ventrally and longer tail. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Tail rounded, dorsally convex conoid, ventrally slightly concave with broad blunt terminus and thickened outer cuticular layer. Spicules arcuate, robust, *ca* 2 times longer than tail length, lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal supplements and 14–16 midventral supplements.

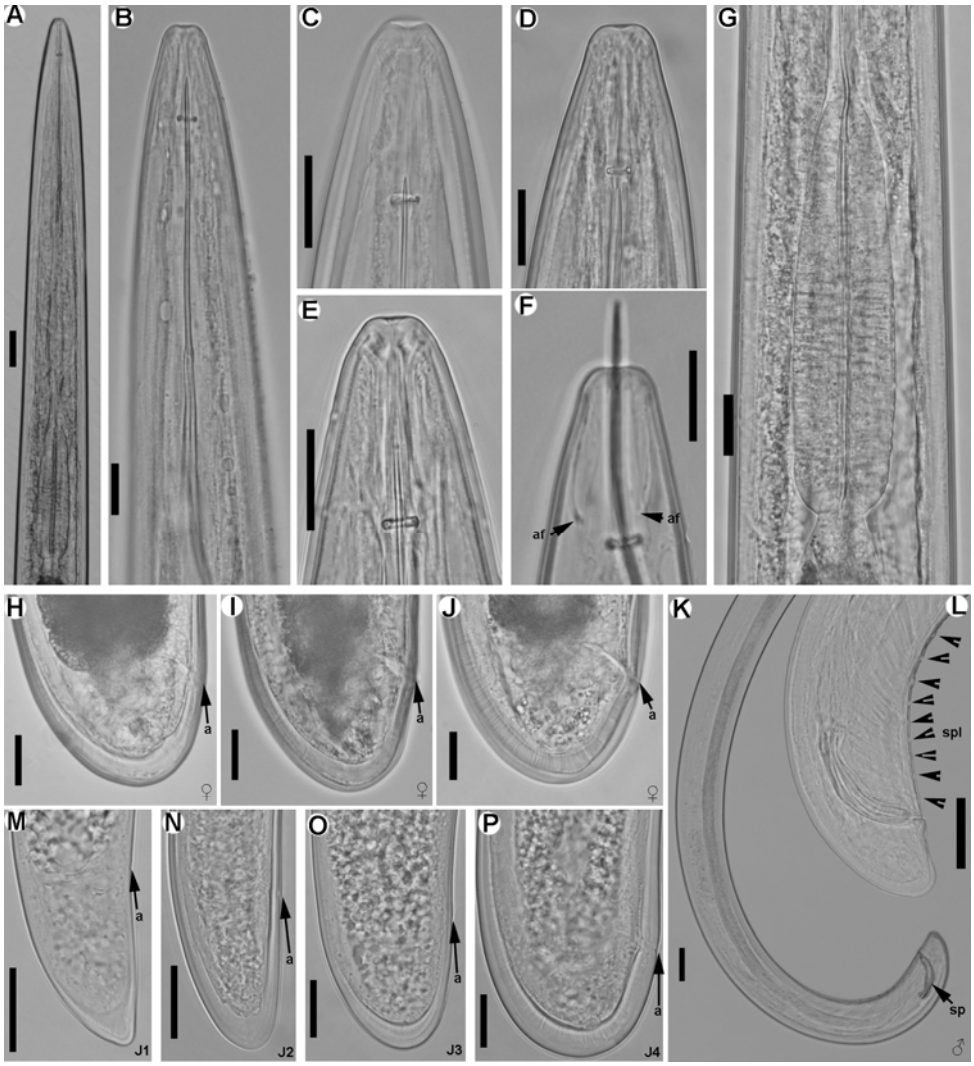

Figura 6.8: Light micrographs of *Longidorus onubensis* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B) Female anterior region. C-F) Female lip regions. G) Detail of basal bulb. H-J) Female tails. K, L) Male tail with detail of spicules. M-P) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; af = amphidial fovea; spl = ventromedian supplements. Scale bars A-J, M-P = 20 µm; K, L = 10 µm.

Table 6.7 Morphometrics of females, males and juvenile stages of *Longidorus onubensis* sp. nov. from the rhizosphere of cultivated olive at Niebla (Huelva province) southern Spain[a].

Characters/ratios[b]	Holotype	Paratype		juvenile-stages			
		Females	Males	J1	J2	J3	J4
n		14	6	6	5	5	5
L (mm)	7.4	8.7 ± 0.68 (7.4-9.6)	8.3 ± 0.80 (7.0-9.3)	2.18 ± 0.20 (1.94-2.42)	2.60 ± 0.20 (2.36-2.86)	4.07 ± 0.90 (3.92-4.16)	5.84 ± 0.92 (4.86-7.16)
a	75.9	88.6 ± 8.4 (75.9-107.5)	96.3 ± 16.8 (69.8-118.9)	63.8 ± 2.6 (60.5-67.9)	62.5 ± 3.6 (58.1-65.8)	64.5 ± 8.0 (50.9-71.1)	77.3 ± 11.9 (62.0-91.9)
b	14.3	17.3 ± 2.0 (14.2-20.9)	17.2 ± 2.6 (14.6-21.2)	9.5 ± 2.2 (6.1-12.1)	9.4 ± 1.8 (7.3-11.6)	12.1 ± 0.9 (10.7-13.0)	12.5 ± 2.5 (11.0-16.7)
c	230.1	211.7 ± 22.9 (184.4-272.7)	178.5 ± 19.5 (150.0-206.7)	56.1 ± 9.8 (47.6-75.2)	67.0 ± 7.4 (60.9-76.7)	95.2 ± 15.6 (78.9-121.0)	139.2 ± 24.2 (105.7-172.5)
c´	0.6	0.7 ± 0.1 (0.6-0.8)	0.8 ± 0.0 (0.7-0.8)	1.6 ± 0.1 (1.5-1.8)	1.3 ± 0.1 (1.1-1.5)	1.0 ± 0.1 (0.8-1.1)	0.8 ± 0.1 (0.7-0.8)
V	51.0	49.7 ± 1.8 (46.0-52.0)	-	-	-	-	-
Odontostyle	121.0	112.9 ± 6.2 (103.0-121.0)	114.7 ± 8.2 (105.0-123.5)	57.7 ± 1.8 (55.5-59.5)	66.6 ± 5.2 (59.0-71.0)	79.9 ± 1.1 (78.5-81.0)	98.8 ± 11.0 (85.5-114.5)
Odontophore	58.0	59.4 ± 5.5 (54.5-72.0)	56.6 ± 7.1 (47.0-65.0)	34.6 ± 4.8 (30.0-41.0)	47.5 ± 8.0 (38.5-54.0)	42.1 ± 7.5 (34.0-53.0)	48.6 ± 6.0 (40.5-54.5)

Characters/ratios[b]	Holotype	Paratype		juvenile-stages			
		Females	Males	J1	J2	J3	J4
Replacement odontostyle	-	-	-	68.4 ± 5.1 (63.5-77.5)	80.6 ± 2.8 (77.0-84.5)	89.7 ± 2.1 (88.0-93.0)	114.4 ± 10.1 (104.5-129.5)
Lip region diam.	15.5	15.4 ± 0.8 (14.0-16.5)	15.3 ± 0.7 (14.5-16.5)	8.0 ± 0.5 (7.5-8.5)	9.3 ± 1.0 (8.5-10.5)	10.6 ± 0.8 (10.0-12.0)	12.4 ± 1.2 (11.5-14.0)
Oral aperture-guiding ring	40.5	33.7 ± 3.1 (28.5-38.5)	40.0 ± 3.1 (35.5-43.5)	21.1 ± 1.5 (19.5-23.0)	23.0 ± 0.8 (22.0-24.0)	27.0 ± 1.3 (25.5-29.0)	33.2 ± 1.6 (31.0-35.5)
Tail length	32.0	41.4 ± 4.7 (32.0-48.5)	46.7 ± 3.0 (42.0-51.0)	39.5 ± 5.9 (31.0-45.5)	39.2 ± 4.9 (34.5-47.0)	43.6 ± 6.8 (32.5-51.0)	42.1 ± 2.7 (39.5-46.0)
Spicules	-	-	94.3 ± 2.1 (92.0-98.0)	-	-	-	-
Lateral accessory piece	-	-	23.6 ± 2.1 (21.0-26.5)	-	-	-	-
J	12.5	13.8 ± 1.3 (12.0-16.0)	14.9 ± 0.9 (14.0-16.5)	9.3 ± 1.4 (7.0-10.5)	7.0 ± 1.0 (6.5-8.5)	8.8 ± 1.0 (7.5-10.0)	10.6 ± 0.7 (10.0-11.5)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Description of juveniles:

Morphologically similar to adults, but smaller. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 6.7, Figures 6.4, 6.7 and 6.8; Robbins *et al.* 1995, 1996). J1s were characterised by a conoid-rounded tail, curved dorsally and slightly concave ventrally with a dorsal-ventral depression at hyaline region level, c' ratio ≥ 1.5 (Table 6.7), an odontostyle length *ca* 58 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages.

Measurements, morphology and distribution:

Morphometric variability is described in Table 7 and morphological traits in Figures 6.4, 6.7 and 6.8. *Longidorus onubensis* sp. nov. was only found in the type locality from the rhizosphere of cultivated olive (Table 6.1, Figure 6.1).

Relationships:

According to the polytomous key by Chen *et al*. (1997) and the supplement by Loof and Chen [65], and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), D (lip region shape), F (body length), and H (tail shape), *L. onubensis* sp. nov. is closed to *L. goodeyi* Hooper 1961, *L. iuglandis* Roca, Lamberti and Agostinelli 1984, *L. oleae* and *L. vinearum*. From *L. goodeyi* it differs mainly in having a longer body and odontostyle length (7.4–9.6 vs 5.6–7.7 mm, 103–121 vs 96–109 µm; respectively), higher c ratio (184.4–272.7 vs 99.0–188.0), and presence vs absence of males) (Hooper 1961, Lamberti *et al.* 1982). On the other hand, from *L. iuglandis* it differs mainly by a slightly longer body length (7.4–9.6 vs 5.4–8.3 mm) (Roca *et al.* 1984, Gutiérrez-Gutiérrez *et al.* 2013). From *L. oleae* it differs mainly by a smaller distance between guiding-ring from anterior end (28.5–38.5 vs 36.0–46.0 µm), a slightly narrower lip region width (14.0–16.5 vs 14.5–21.0 µm), and amphidial fovea shape (symmetrically vs asymmetrically bilobed) (Gutiérrez-Gutiérrez *et al.* 2013)). Finally, *L. onubensis* sp. nov. differs mainly from *L. vinearum* in having a slightly smaller distance between guiding-ring from anterior end and spicules length (28.5–38.5 vs 32.5–47.0 µm, 92.0–98.0 vs 100.0–136.5 µm;

respectively), and narrower lip region width (14.0–16.5 vs 18.0–28.0 µm) (Table 6.12; (Bravo and Roca 1995, 1998)).

Molecular divergence of the new species:

D2–D3 region of *L. onubensis* sp. nov. (KT308857-KT308858) was 95 and 94% similar to L. goodeyi (AY601581) and *L. vinearum* (KT308874-KT308877), respectively (Table 6.5). Intraspecific variation of D2–D3 segments detected amongst the studied individuals, consisted of one nucleotide and no indels (99% similarity). Similarly, intraspecific variation of the ITS1 for these sequences (KT308882-KT308883) was low, 99% similarity with 0 nucleotides differences and 3 gaps. ITS1 also showed some similarity (85%) with *L. vinearum* (KT308892-KT308893). Finally, the partial 18S of *L. onubensis* sp. nov. (KT308897) showed a high level of similarity (99%) with *L. oleae* (JX445119), *L.vineacola* (JX445123), and *L. andalusicus* (JX445118).

3.2.4 *Longidorus silvestris* sp. nov.

urn:lsid:zoobank.org:pub:C8230A9D-FD45-4AA4-9ABF-8445E8001CCC

Holotype

Adult female, collected from the rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) (36°06'34.4"N latitude, 5°42'39.5"W longitude), Tarifa, Cádiz province, Spain; collected by P. Castillo, May 1, 2012; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR27-19).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and

deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers AR27-01-AR27-15); one female at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (AR27-16); one female at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT838); and two females at USDA Nematode Collection, Beltsville, MD, USA (T-6632p); collected by P. Castillo, May 1, 2012.

Diagnosis

Longidorus silvestris sp. nov. is a gonochoristic species characterized by a long and robust body (5.0–7.0 mm), assuming an open C-shaped when heat relaxed; lip region narrow, conoid-rounded, continuous with body contour, 9.5–11.5 µm wide; guiding-ring located 30.5–35.5 µm from anterior end; odontostyle 76.0–89.0 µm long; amphidial fovea pocket-shaped symmetrically bilobed; vulva equatorial; female tail short, hemispherical to blunty-conoid, bearing two or three pairs of caudal pores and c' ratio (0.7–1.0); male extremely rare, only one male found, with spicules 69 µm long and 11 ventromedian supplements; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (GenBank accession numbers KT308859-KT308860, KT308884, and KT308898, respectively). According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A3(2)-B1-C3-D1-E2-F3-G2(1)-H1-I12.

Etymology

The species name refers to the habitat (*silvestris*, *silvestre* = sylvan, living in the wild forest), where the type specimens were collected.

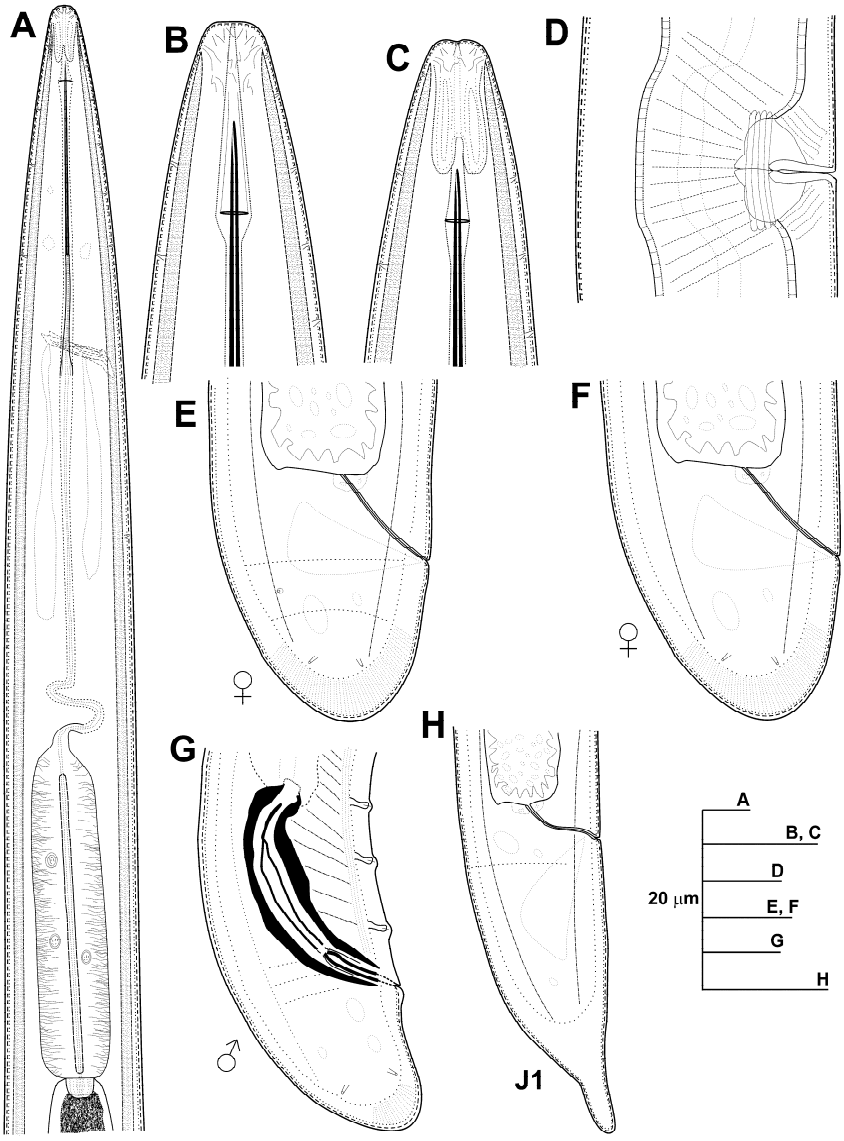

Figura 6.9: Line drawings of *Longidorus silvestris* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E, F) Female tails. G) Male tail. H) First-stage juvenile tail (J1).

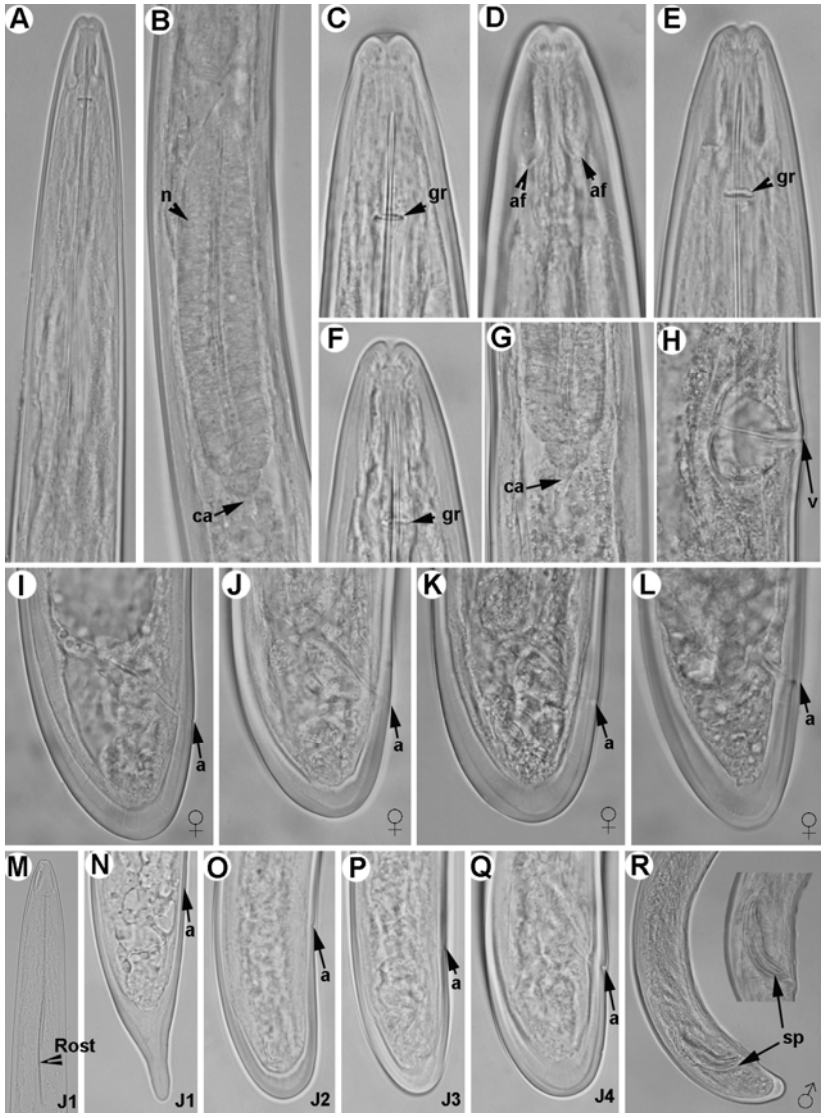

Figura 6.10: Light micrographs of *Longidorus silvestris* sp. nov., female paratypes, male and juvenile stages. A) Female anterior region. B) Detail of basal bulb. C-F) Female lip regions. G) Detail of pharyngealintestinal junction. H) Vulval region. I-L) Female tails. M) First-stage juvenile lip region showing replacement odontostyle inside odontophore. N-Q) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. R, S) Male tail and detail of spicules. Abbreviations: a = anus; af = amphidial fovea; ca = cardias; gr = guiding-ring; n = nucleus; Rost = replacement odontostyle; sp = spicules. Scale bars = 20 µm.

Table 6.8 Morphometrics of females, males and juvenile stages of *Longidorus silvestris* sp. nov. from the rhizosphere of wild olive at Tarifa (Cádiz province) southern Spain[a].

Characters/ratios[b]	Holotype	Paratype		juvenile-stages			
		Females	Male	J1	J2	J3	J4
n		19	1	7	6	5	5
L (mm)	6.0	5.8 ± 0.51 (5.0-7.0)	5.8	1.66 ± 0.27 (1.19-2.03)	2.43 ± 0.24 (2.16-2.84)	3.13 ± 0.52 (2.53-3.78)	4.75 ± 0.51 (4.15-5.39)
a	93.5	86.9 ± 7.5 (73.0-101.4)	96.6	62.4 ± 5.4 (56.9-70.4)	73.6 ± 4.8 (67.5-80.0)	76.9 ± 6.1 (69.3-85.4)	91.7 ± 5.6 (86.2-99.4)
b	15.0	14.2 ± 1.5 (11.2-16.3)	12.8	7.9 ± 1.5 (5.8-10.2)	8.4 ± 1.2 (6.5-10.0)	9.4 ± 1.8 (7.8-11.8)	11.9 ± 2.1 (10.2-15.3)
c	145.2	153.9 ± 12.1 (136.9-182.4)	143.1	35.3 ± 6.6 (50.6-54.8)	57.3 ± 4.2 (50.5-61.0)	82.0 ± 13.5 (67.6-99.6)	120.4 ± 8.2 (110.6-133.0)
c´	0.9	0.8 ± 0.1 (0.7-1.0)	0.9	2.5 ± 0.3 (2.0-2.8)	1.7 ± 0.1 (1.6-1.9)	1.3 ± 0.1 (1.2-1.5)	1.0 ± 0.1 (0.9-1.1)
V	50	50.1 ± 0.6 (49.0-51.5)	-	-	-	-	-
Odontostyle	86.5	82.7 ± 3.4 (76.0-89.0)	83.5	48.7 ± 3.4 (44.0-52.0)	56.1 ± 2.3 (53.0-58.0)	64.3 ± 2.6 (61.5-67.5)	75.8 ± 2.0 (74.0-79.0)
Odontophore	49.0	50.9 ± 5.0 (42.0-62.0)	42.0	33.3 ± 2.1 (30.0-36.0)	34.3 ± 1.3 (32.0-36.0)	37.9 ± 3.8 (33.5-43.0)	43.5 ± 3.0 (40.0-48.0)

		Paratype		juvenile-stages			
Characters/ratios[b]	**Holotype**	**Females**	**Male**	**J1**	**J2**	**J3**	**J4**
Replacement odontostyle	-	-	-	58.1 ± 4.7 (50.5-65.0)	65.7 ± 2.7 (62.0-70.0)	77.4 ± 3.9 (70.5-80.0)	85.2 ± 3.3 (82.0-89.5)
Lip region diam.	10.0	10.2 ± 0.6 (9.5-11.5)	10.5	7.1 ± 0.3 (6.5-7.5)	7.3 ± 0.3 (7.0-7.5)	7.4 ± 0.4 (7.0-8.0)	9.5 ± 0.4 (9.0-10.0)
Oral aperture-guiding ring	31.0	32.3 ± 1.6 (30.0-35.5)	33.0	18.6 ± 1.1 (16.5-20.0)	23.0 ± 1.0 (21.5-24.0)	24.5 ± 1.4 (23.0-26.5)	30.4 ± 1.7 (29.5-33.5)
Tail length	41.0	37.9 ± 2.3 (33.0-42.0)	40.5	47.5 ± 6.7 (42.0-61.0)	42.6 ± 4.7 (35.5-46.5)	38.1 ± 0.4 (37.5-38.5)	39.4 ± 2.1 (37.5-42.5)
Spicules	-	-	69.0	-	-	-	-
Lateral accessory piece	-	-	17.0	-	-	-	-

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Description of taxa

Female:

Body robust, slightly tapering towards anterior end, usually assuming an open C-shaped when heat relaxed. Cuticle appears smooth, 3.2 ± 0.2 (3.0–3.5) µm thick, 13.6 ± 4.3 (8.0–19.0) µm thick at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region narrow, conoid-rounded, continuous with body contour. Amphidial fovea pocket-shaped symmetrically bilobed, extending about 3/4 part of anterior end-guiding ring distance. Labial papillae prominent. Guiding system with well-developed compensation sacs. Stylet guiding-ring single, located at 32.3 ± 1.6 (30.0–35.5) µm from anterior end. Odontostyle moderately long and narrow, 1.6 ± 0.2 (1.4–2.0) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling narrower part of pharynx. Pharynx consisting of an anterior slender narrow part 307–572 µm long, extending to a cylindrical, terminal pharyngeal bulb, well demarcated anteriorly, 103–155 µm long and occupying *ca* 22–40% of total pharyngeal length. Glandularium 110.6 ± 13.2 (92.0–136.55) µm long. Dorsal pharyngeal gland nucleus (DN) located at 35.3 ± 4.4 (28.4–42.2)%, nucleolus being slightly larger (2.0–4.5 vs 2.5–3.5 µm) than nucleoli of two ventrosublateral nuclei (SVN) situated at 57.8 ± 4.0 (53.2–64.4)% of distance from anterior end of pharyngeal bulb, respectively. Cardia well-developed, hemispherical, 17.3 ± 2.6 (15.0–21.0) µm long. Reproductive system with both genital branches equally developed, 7.9 ± 0.8 (6.1–9.3), 7.9 ± 0.9 (6.4–9.9)% of body length, respectively. Ovaries reflexed, variable in length, *ca* 72–110 µm long. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, 24.5 ± 2.6 (18.5–32.0) µm long, or 28–48% of corresponding max body width, surrounded by well-developed muscles. Uteri 456 ± 52.7 (372–578) µm long, without sperm cells in the female specimens examined and well-developed sphincter between uterus and oviduct. Prerectum short and variable in length, 414.3 ± 79.9 (266.0–489.0) µm long or *ca* 5–9% of body length. Rectum 31.4 ± 4.1 (26.5–37.0) µm long. Tail short, hemispherical to blunty-conoid shape, bearing two or three pairs of caudal pores.

Male:

Extremely rare, only one male specimen was found. Morphologically similar to female except for genital system and posterior region slightly curved ventrally. Tail convex-conoid, ventrally slightly concave with broad blunt terminus and the thickened outer cuticular layer. Male genital tract diorchic with test opposed, containing multiple rows of different stages of spermatogonia. Spicules arcuate, robust, about 2 times longer than tail length, lateral guiding pieces more or less straight. One pair of adanal supplements preceded by a row of 10 ventromedian supplements.

Description of juveniles:

Morphologically similar to adults in most respects except for size and development reproductive system. All juvenile developmental stages were detected and distinguished by relative lengths of body and functional and replacement odontostyle (Table 6.8, Figures 6.4 and 6.10; Robbins *et al.* 1995, 1996), and the genital primordium. J1s characterised by a conoid-rounded tail, slightly curved dorsally and dorsal-ventral depression at hyaline region level, subdigitate (Figure 6.10), with a c´ ratio *ca* 2.5, odontostyle length *ca* 49 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages. However, morphology in second-, third- and fourth-stages (except for undeveloped genital structures) similar to that of female, including broadly conoid to hemispherical tail shape with rounded terminus, which becoming progressively shorter and stouter in each moult and shorter distance from anterior end to guiding-ring in each moult (Figure 6.10).

Measurements, morphology and distribution:

Morphometric variability is described in Table 6.8 and morphological traits in Figures 6.9 and 6.10. *Longidorus silvestris* sp. nov. was only found in type locality from the rhizosphere of wild olive (Table 6.1, Figure 6.1).

Relationships:

On the basis of body and odontostyle length, distance between guiding-ring from anterior body end, a, c and c´ ratios, amphidial fovea, or female tail shape, *L. silvestris* sp. nov. is very closely related to *L. wicuolea* sp. nov. from which it can be differentiated by a combination of these characters,

but particularly in lip region shape (continuous vs separated from body contour by slight depression), and J1 tail shape (conoid-subdigitate vs conoid) (Figures 6.9 and 6.10). In addition, according to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), F (body length), and H (tail shape), *L. silvestris* sp. can be related with *L. belloi* Andrés and Arias 1988, *L. igoris* Krnjaić, Lamberti, Krnjaić, Agostinelli and Radicci 2000, and L. moesicus Lamberti, Choleva and Agostinelli 1983. From *L. belloi* it differs mainly in having a slightly shorter odontostyle length (76.0–89.0 vs 74.8–101.7 µm), slightly higher c´ ratio (0.7–1.0 vs 0.5–1.1), frequency of males (extremely rare vs common), amphidial fovea shape (symmetrically vs asymmetrically bilobed) and J1 tail shape (conoid-subdigitate vs conoid) (Arias and Andres 1988, Bravo and Roca 1998). On the other hand, *L. silvestris* sp. nov. differs mainly from *L. igoris* by lower a ratio (73.0–101.4 vs 103.0–131.7) and J1 tail shape (conoid-subdigitate vs cylindrical) (Krnjaic *et al.* 2000). Finally, the new species differs mainly from *L. moesicus* in having lower a ratio (73.0–101.4 vs 96.0–147.0) and a shorter odontostyle length (76.0–89.0 vs 97.0–124.0 µm) (Lamberti *et al.* 1983, Tzortzakakis *et al.* 2014).

Molecular divergence of the new species:

Longidorus silvestris sp. nov. was closely related in D2–D3 (KT308859-KT308860) to L. wicuolea sp. nov. (KT308863-KT308866) with 98% similarity (Table 6.5). Intraspecific variation of D2–D3 detected between the two studied populations was low, 6 nucleotides and no indels. ITS1 also agree with the results obtained for D2–D3, this sequence (KT308884) was 90% similar to *L. wicuolea* sp. nov. (KT308887-KT308889). Finally, the partial 18S (KT308898) showed high homology with several sequences deposited in the GenBank, such as *L. magnus* (HM92921345, KT308902), *L. vinearum* (KT308903), *L. lusitanicus* (KT308901) and *L. wicuolea* sp. nov. (KT308900).

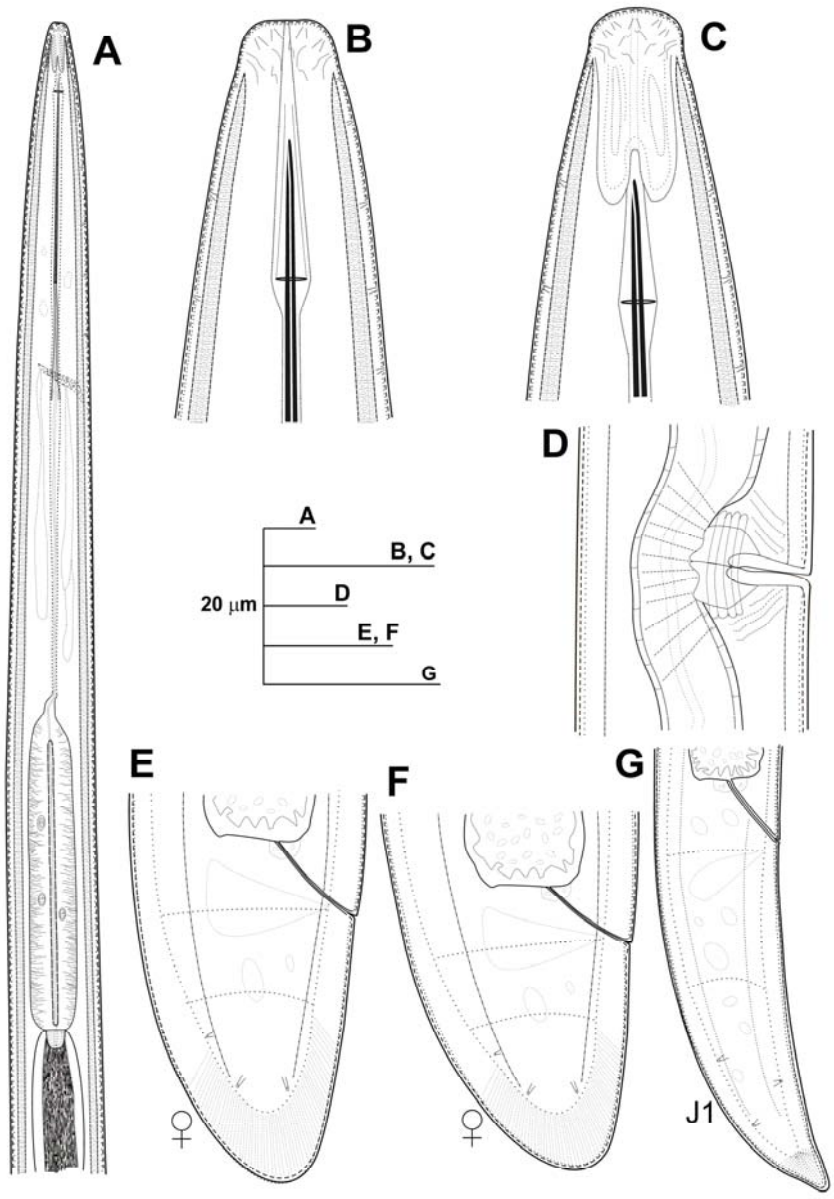

Figura 6.11: Line drawings of *Longidorus vallensis* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E, F) Female tails. G) First-stage juvenile tail (J1).

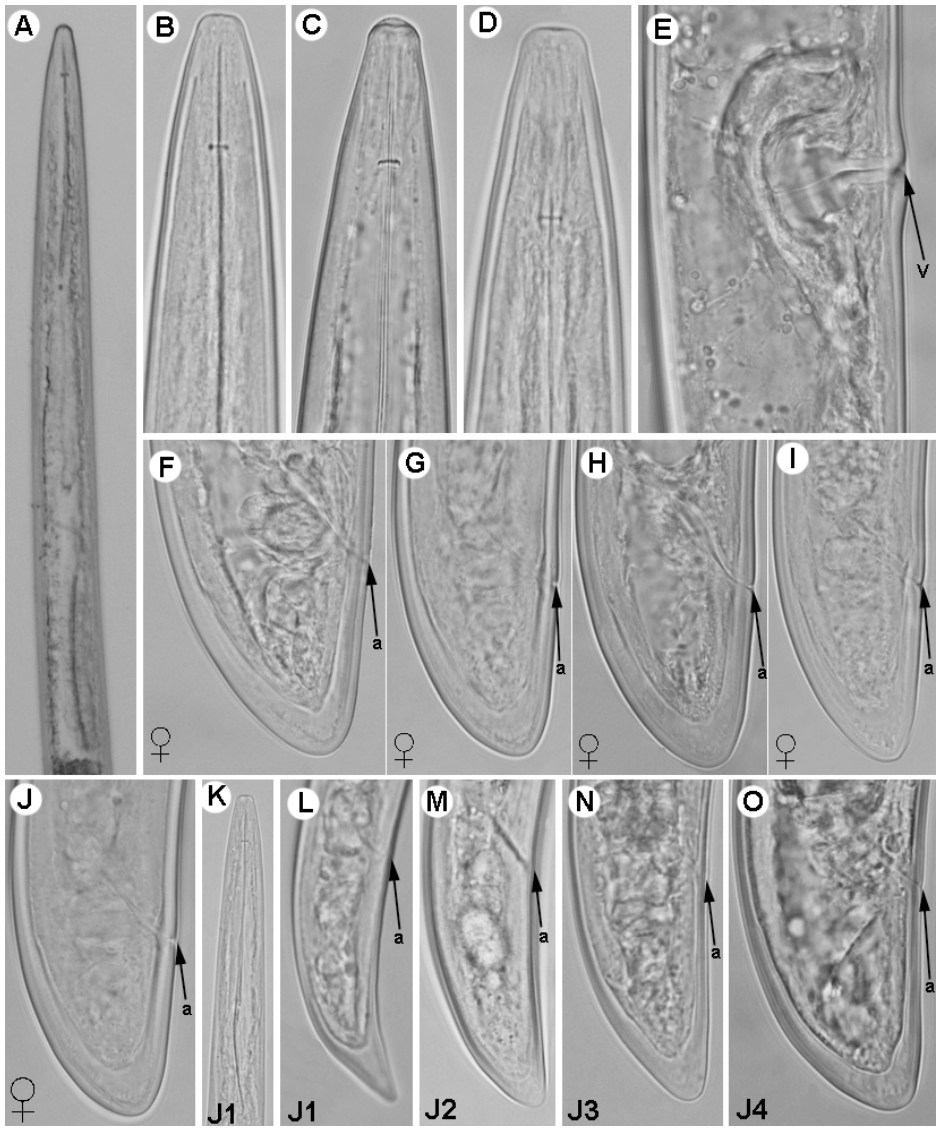

Figura 6.12: Light micrographs of *Longidorus vallensis* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B-D) Female lip regions. E) Vulval region. F-J) Female tails. K) First-stage juvenile lip region showing replacement odontostyle inside odontophore. L-O) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. R) Male tail with detail of spicules. Abbreviations: a = anus; af = amphidial fovea; ca = cardias; gr = guiding-ring; n = nucleus; Rost = replacement odontostyle; sp = spicules. Scale bars = 20 µm.

3.2.5 *Longidorus vallensis* sp. nov.

urn:lsid:zoobank.org:act:9C1B1CB3-F8CE-422B-BAE1-984B1BFE2173

Holotype

Adult female, collected from the rhizosphere of wild olive (*Olea europaea* subsp. *silvestris* (Miller) Lehr) (36°37'57.3"N, 005°46'20.0"W), at San José del Valle, Cádiz province, Spain; collected by A. Archidona-Yuste, March 17, 2013; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number AR55-16).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers AR55-01-AR55-13); two females at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (AR55-14); two females at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT8340); and two females at USDA Nematode Collection, Beltsville, MD, USA (T-6633p); collected by A. Archidona-Yuste, March 17, 2013.

Diagnosis

Longidorus vallensis sp. nov. is characterized by a long and thin body (6.2–8.7 mm), assuming an open C-shaped when heat relaxed; lip region anteriorly rounded separated from body contour by slight depression, 9.0–10.0 µm wide; guiding-ring located 25–30 µm from anterior end; odontostyle moderately long and narrow (71.5–85.0 µm); amphidial fovea pocket-shaped slightly symmetrically bilobed; vulva almost equatorial; female relatively tail short, convex-conoid to bluntly conoid, and bearing

three pairs of caudal pores; c' ratio (1.0–1.4); males not found; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (Gen-Bank accession numbers KT308861-KT308862, KT308885-KT308886, and KT308899, respectively). According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A2(3)-B1-C2-D2-E2-F4(3)-G3-H2-I1.

Etymology

The species epithet refers to San José del Valle, the name of the type locality, Cádiz province, where the type specimens were collected.

Description of taxa

Female:

Body long and thin, almost cylindrical, tapering in both extremities, especially in the anterior end. When heat relaxed, body usually assuming a spiral to an open C-shaped. Cuticle appearing smooth under low magnifications, 2.5 ± 0.8 (1.5–4.5) µm thick at mid body, but thicker (5.7 ± 1.4 (3.5–7.5) µm) and marked by very fine superficial transverse striate mainly in tail region, as shown by higher magnifications. Lip region anteriorly rounded, separated from body contour by slight depression. Amphidial fovea pocket-shaped slightly symmetrically bilobed. Labial papillae prominent. Stylet guiding-ring single, located 2.8 ± 0.1 (2.6–3.0) times lip region diam. from anterior end. Lateral chord 13.9 ± 1.9 (12.0–16.0) µm wide at mid-body or 20–30% of corresponding body diam. Odontostyle moderately long and narrow, straight or slightly arcuate, 1.8 ± 1.9 (1.5–2.0) times as long as odontophore, *ca* 3.0–3.5 µm wide towards its base; odontophore weakly developed, with rather weak basal swellings. Nerve ring encircling cylindrical part of pharynx, 2.2 ± 0.2 (1.9–2.7) times body width at neck base far from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb relatively long and cylindrical, 118.5 ± 8.0 (106.5–135.0) µm long or ca one-third of neck length, 18.4 ± 2.0 (16.0–22.5) µm diam. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral pair of nuclei (SN) situated slightly posterior to normal arrangement of pharyngeal glands (Chen *et al.* 1997, Loof and

Chen 1999), 34.1 ± 4.8 (27.8–40.7)%, 57.8 ± 5.0 (52.1–69.7)% of distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nucleus (DN) slightly larger than nuclei of two SVN (2.5–3.5 vs 1.5–2.5 µm in diam.). Glandularium 102.3 ± 4.9 (95.0–113.0) µm long. Cardia conoid-rounded, 9.2 ± 2.6 (7.0–12.0) µm long. Reproductive system with both genital branches equally developed, very short compared with body length, ranging between 335–597 µm long, with reflexed ovaries variable in length. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, 23.0 ± 4.6 (16.0–30.0) µm long, or 30–50% of corresponding body width, surrounded by well-developed muscles. Genital branches equally developed, 5.6 ± 1.3 (4.3–8.2), 6.0 ± 1.3 (4.8–8.3)% of body length, respectively. Uteri short, without sperm cells in the female specimens examined. Anterior and posterior oviduct of similar size. Ovaries equally developed, 106–147 µm long, both of them with a single row of oocytes. Prerectum variable in length, 984.1 ± 133.2 (800–1194) µm long, and rectum 27.2 ± 2.6 (23.5–32.0) µm long, anus a small rounded slit. Tail relatively short, convex-conoid to bluntly conoid, with rounded terminus, bearing three pairs of caudal pores.

Male:

Not found.

Description of juveniles:

Morphometrics obtained from juvenile specimens, and of the relative lengths of body, tail, and functional and replacement odontostyle, confirmed the presence of four juvenile stages (Table 6.9, Figures 6.4 and 6.12; Robbins *et al.* 1995, 1996). J1s were characterised by a conoid tail, dorso-ventrally curved with rounded terminus, and slightly depression at hyaline region level, c' ratio ≥ 2.3 (Table 6.9); an odontostyle length *ca* 53 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages.

Table 6.9 Morphometrics of females, males and juvenile stages of *Longidorus vallensis* sp. nov. from the rhizosphere of cultivated and wild olives at several localities (Cádiz and Córdoba provinces) southern Spain[a].

Host/locality, sample code	**wild olive, San José del Valle (Cádiz province), AR55**						**cultivated olive Cabra (Córdoba province), M0012**
Characters/ratios[b]	**Holotype**	**Paratype Females**	**J1**	**J2**	**J3**	**J4**	**Female**
n		17	5	5	5	5	1
L (mm)	7.0	7.6 ± 0.61 (6.2-8.7)	1.56 ± 0.57 (1.48-1.64)	2.66 ± 0.43 (2.23-3.34)	4.09 ± 0.50 (3.55-4.84)	5.86 ± 0.36 (5.41-6.39)	7.94
a	145.1	135.5 ± 6.2 (125.1-145.3)	74.5 ± 2.9 (71.0-77.6)	85.6 ± 5.0 (79.7-92.8)	108.0 ± 7.7 (100.4-118.9)	132.9 ± 8.5 (122.3-142.4)	149.8
b	16.1	18.6 ± 2.9 (12.6-23.6)	6.8 ± 1.4 (4.5-7.9)	10.1 ± 2.8 (6.1-13.9)	14.1 ± 3.5 (10.5-19.3)	17.6 ± 2.1 (14.5-19.8)	22.5
c	189.7	181.1 ± 18.6 (126.6-208.5)	38.4 ± 3.1 (34.9-43.4)	63.0 ± 4.0 (59.7-69.6)	87.4 ± 8.5 (81.3-99.8)	130.0 ± 11.7 (113.9-146.8)	198.5
c´	1.1	1.2 ± 0.1 (1.0-1.4)	2.6 ± 0.2 (2.3-2.7)	2.0 ± 0.1 (1.8-2.0)	1.7 ± 0.1 (1.6-1.8)	1.4 ± 0.1 (1.3-1.5)	1.1
V	53.5	51.4 ± 1.4 (49.5-53.5)	-	-	-	-	51.0
Odontostyle	80.5	79.1 ± 3.5 (71.5-85.0)	53.2 ± 1.6 (50.5-55.0)	55.2 ± 1.1 (54.5-57.0)	62.9 ± 1.4 (61.0-64.5)	73.3 ± 2.5 (69.5-76.0)	84.0

Host/locality, sample code	wild olive, San José del Valle (Cádiz province), AR55						cultivated olive Cabra (Córdoba province), M0012
Characters/ratios[b]	Holotype	Paratype Females	J1	J2	J3	J4	Female
Odontophore	47.0	45.7 ± 3.2 (40.5-54.0)	26.8 ± 2.5 (24.5-29.5)	32.6 ± 6.9 (20.5-37.0)	45.4 ± 4.5 (40.5-51.0)	41.9 ± 6.8 (31.5-47.5)	56.0
Replacement odontostyle	-	-	59.3 ± 2.1 (57.0-62.0)	65.1 ± 1.9 (62.0-67.0)	73.5 ± 1.5 (71.5-75.5)	80.1 ± 3.6 (76.0-83.5)	-
Lip region diam.	10.0	9.7 ± 0.3 (9.0-10.0)	7.0 ± 0.7 (6.0-7.5)	7.9 ± 0.4 (7.5-8.5)	8.4 ± 0.2 (8.0-8.5)	9.1 ± 0.7 (8.5-10.0)	10.5
Oral aperture-guiding ring	28.0	27.3 ± 1.3 (25.0-30.0)	18.7 ± 1.5 (17.0-21.0)	22.0 ± 0.8 (21.0-23.0)	23.2 ± 1.3 (22.0-25.0)	26.5 ± 1.4 (25.0-28.5)	27.0
Tail length	42.5	42.0 ± 2.9 (37.5-49.0)	40.9 ± 2.8 (36.0-42.5)	42.1 ± 5.0 (35.0-48.0)	46.7 ± 1.9 (43.5-48.5)	45.2 ± 1.7 (43.5-47.5)	40
J	11.0	10.3 ± 1.1 (8.0-12.0)	11.6 ± 1.2 (9.5-12.5)	8.2 ± 0.6 (7.5-8.5)	8.5 ± 1.6 (6.5-10.5)	9.8 ± 1.6 (8.0-12.0)	10

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; J (hyaline tail region length).

Measurements, morphology and distribution:

Morphometric variability is described in Table 6.9 and morphological traits in Figures 6.4, 6.11 and 6.12. In addition to the type locality, *L. vallensis* sp. nov. was found from one cultivated olive sample located in Córdoba province (Table 6.1, Figure 6.1).

Relationships:

According to the polytomous key by Chen *et al*. (1997) and the supplement by Loof and Chen (1999), and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (lip region shape), F (body length), and H (tail shape), *L. vallensis* sp. nov. groups with *L. belloi*, *L. tabrizicus* Niknam *et al*. 2010 and *L. wicuolea* sp. nov. From *L. belloi* it differs mainly in having higher a and c´ ratio (125.1–149.8 vs 73.0–132.0, 1.0–1.4 vs 0.5–1.1; respectively), and the absence vs presence of males (Arias and Andres 1988, Bravo and Roca 1998). On the other hand, *L. vallensis* sp. nov. differs from *L. tabrizicus* mainly by a longer body and odontostyle length (6.2–8.7 vs 4.1–6.1 mm, 71.5–85.0 vs 61.5–70.0 µm; respectively), higher a and c ratio (125.1–149.8 vs 81.5–135.0, 126.6–208.5 vs 91.0–155.0; respectively), and the absence vs presence of males (Niknam *et al.* 2010). Finally, from *L. wicuolea* sp. nov. differs mainly in having higher a ratio (125.1–149.8 vs 79.3–115.6) and slightly higher c´ ratio (1.0–1.4 vs 0.8–1.2) (Table 6.10, Figures 6.13 and 6.14). In addition, *L. vallensis* sp. nov. is molecularly related to *L. rubi* from which it can be mainly differentiated by a longer body length (6.2–8.7 vs 4.0–6.0 mm), higher c ratio (126.6–208.5 vs 70.0–126.9) and lower c´ ratio (1.0–1.4 vs 1.7–2.1) (Romanenko 1998, Gutiérrez-Gutiérrez *et al.* 2013).

Molecular divergence of the new species:

The sequence divergence between *L. vallensis* sp. nov. (KT308861-KT308862) and other congeneric species were significant. The closet species in relation to D2–D3 region were *L. rubi* (JX445116, 96% similarity) and *L. indalus* sp. nov. (KT308852-KT308854, 91% similarity) (Table 6.5). Low intraspecific variation was detected in the two studied populations,

differing in 3 nucleotides and 0 gaps. ITS1 (KT308885-KT308886) also showed some similarity with *L. rubi* (JX445098, 81%). No more similarity values above 80% were found in GenBank. Intraspecific variations for ITS1 sequences were 22 nucleotides, and 4 indels. The partial 18S of *L. vallensis* sp. nov. (KT308899) matched closely, 99%, with several *Longidorus* species, such as *L. rubi* (JX445125), *L. tabrizicus* (FJ009678), *L. closelongatus* (KJ802897) and *L. cretensis* (KJ802898).

3.2.6 *Longidorus wicuolea* sp. nov.

urn:lsid:zoobank.org:act:53950FE4-AA33-4301-AFE7-D143C0FC24AE

Holotype

Adult female, collected from the rhizosphere of cultivated olive (*Olea europaea* subsp. *europaea* L.) (37°28'37.4"N, 005°42'26.7"W), at Carmona, Sevilla province, Spain; collected by A. Archidona-Yuste, May 13, 2015; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number JAO95-17).

Paratypes

Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers JAO95-01-JAO95-16); one female at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (JAO95-18); one female at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RIT841); and two females at USDA Nematode Collection, Beltsville, MD, USA (T-6634p); collected by A. Archidona-Yuste, March 17, 2013.

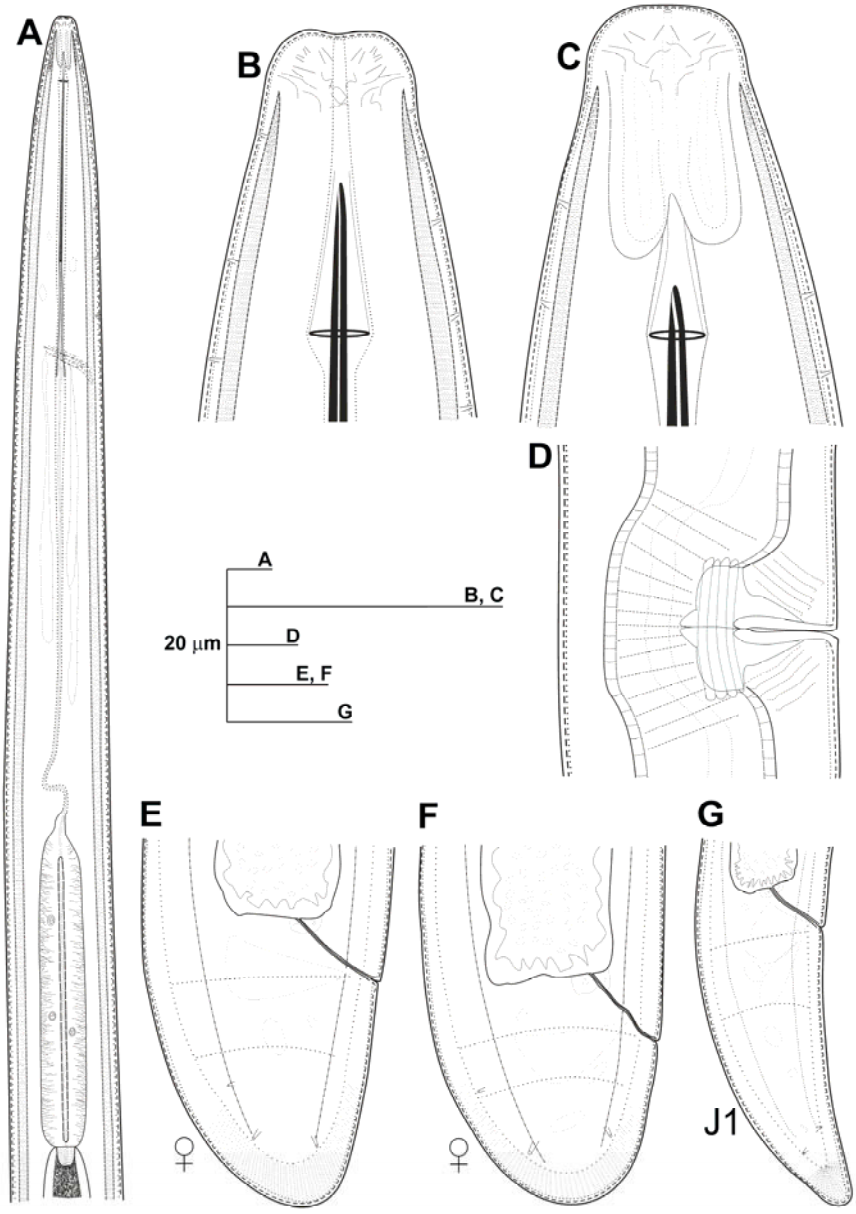

Figura 6.13: Line drawings of *Longidorus wicuolea* sp. nov., female paratypes, male and juvenile stages. A) Pharyngeal region. B, C) Details of lip region. D) Vulval region. E, F) Female tails. G) First-stage juvenile tail (J1).

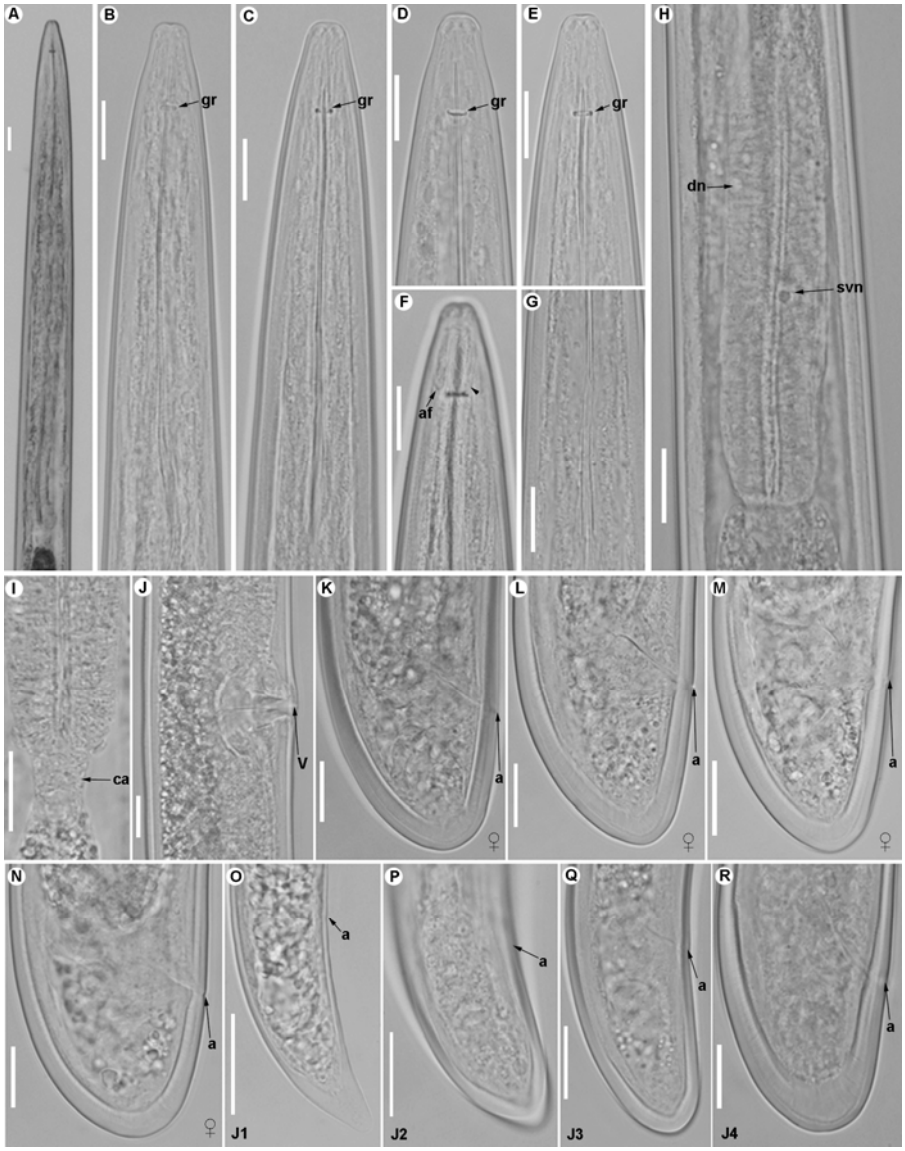

Figura 6.14: Light micrographs of *Longidorus wicuolea* sp. nov., female paratypes and juvenile stages. A) Pharyngeal region. B-C) Female neck regions. D-F) Female lip regions. G) Detail of odontophore. H) Detail of pharyngeal bulb. I) Detail of cardias (pharyngeal-intestinal junction). J) Vulval region. K-N) Female tails. O-R) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; af = amphidial fovea; ca = cardias; gr = guiding-ring; dn = dorsal nucleus; svn = subventral nucleus; V = vulva. Scale bars = 20 µm.

Diagnosis

Longidorus wicuolea sp. nov. is characterized by a long and robust body (6.1–8.7 mm), assuming an open C-shaped when heat relaxed; lip region anteriorly rounded, separated from body contour by a slight depression, 9.5–12.0 µm wide; guiding-ring located 27–33 µm from anterior end; odontostyle moderately long (77–94 µm); amphidial fovea pocket-shaped symmetrically bilobed; vulva almost equatorial; female relatively tail short, convex-conoid to bluntly conoid, and bearing two or three pairs of caudal pores; c' ratio (0.8–1.2); males not detected; and specific D2–D3, ITS1 rRNA and partial 18S rRNA sequences (Gen-Bank accession numbers KT308863-KT308866, KT308887-KT308889, and KT308900, respectively). According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), the new species has the following code (codes in parentheses are exceptions): A3(2)-B1(2)-C32-D2-E2-F4(3)-G2(1)-H12-I1.

Etymology

The species epithet refers to the first letters of its host plants name, wild (*wi*) and cultivated (*cu*) olive (*olea*), where the type specimens were collected.

Description of taxa

Female:

Body long and robust, slightly tapering towards anterior end, usually assuming an open C-shaped when heat relaxed. Cuticle appears smooth, 3.8 ± 0.7 (2.5–5.0) µm thick, 6.5 ± 1.0 (5.5–8.0) µm thick at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region anteriorly rounded, separated from body contour by slight depression. Amphidial fovea pocket-shaped symmetrically bilobed, with lobes of about equal length, and extending about 3/4 part of anterior end-guiding ring distance. Labial papillae prominent. Guiding system with well-developed compensation sacs. Stylet guiding-ring single, located at 29.7 ± 1.6 (27.0–33.0) µm from anterior end. Odontostyle moderately long and narrow, 1.8 ± 0.2 (1.4–2.2) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak basal

swellings. Lateral chord *ca* 19% of corresponding body diam. Nerve ring encircling cylindrical part of pharynx, 1.7 ± 0.2 (1.4–2.0) times body width at neck base far from anterior end. Pharynx consisting of an anterior slender narrow part, extending to a terminal pharyngeal bulb, well demarcated anteriorly and cylindrical, 136.6 ± 9.8 (117.0–150.0) µm long, occupying *ca* 30% of total pharyngeal length, and 28.9 ± 3.0 (23.5–34.5) µm wide. Glandularium 11 ±0.6 ± 13.2 (92.0–136.55) µm long. Normal arrangement of pharyngeal glands [64, 65]: nuclei of the dorsal (DN) and subventral (SVN) pharyngeal gland located at 28.1 ± 2.8 (23.1–31.5), 56.8 ± 3.1 (50.9–61.6)% of distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nucleus (DN) slightly larger than nuclei of two SVN (3.5–5.0 vs 3.0–4.5 µm in diam.). Cardia well developed, hemispherical to conoid, 14.1 ± 1.0 (12.5–15.5) µm long. Reproductive system with both genital branches equally developed, 7.4 ± 1.0 (5.7–9.8), 7.4 ± 0.8 (6.0–9.0)% of body length, respectively. Ovaries reflexed, very variable in length, *ca* 100–205 µm long. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, 30.9 ± 4.1 (20.0–36.0) µm long, or 30–50% of corresponding body width, surrounded by well-developed muscles. Uterus and oviduct of about equal length, without sperm cells in the female specimens examined. Ovaries equally developed *ca* 100–205 µm long, both of them with a single row of oocytes. Prerectum very variable in length, 11.1 ± 3.8 (6.4–16.3) times anal body diam., and rectum 1.6 ± 0.3 (1.3–2.0) times as long as anal body diam., anus a small rounded slit. Tail relatively short, convex-conoid to bluntly-conoid, with rounded terminus, bearing two or three pairs of caudal pores.

Male:

Not detected.

Description of juveniles:

Morphologically similar to adults, but smaller. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 6.10, Figures 6.4 and 6.14, Robbins *et al.* 1995, 1996). J1s were characterised by a conoid tail, dorso-ventrally curved with rounded terminus, and slightly depression at tip tail level, c' ratio ≥ 1.7 (Table 6.10); an odontostyle length *ca* 52 µm, and shorter distance from anterior end to stylet guiding-ring than that in adult stages.

Measurements, morphology and distribution:

Morphometric variability is described in Table 6.10 and morphological traits in Figures 6.4, 6.13 and 6.14. In addition to the type locality, *L. wicuolea* sp. nov. was extracted from one wild olive sample located in Huelva province, being distributed only in Western Andalusia (Table 6.1, Figure 6.1).

Relationships:

On the basis of body and odontostyle length, distance between guiding-ring from anterior body end, a, c and c´ ratios, amphidial fovea, or female tail shape, *L. wicuolea* sp. nov. is very closely related to *L. silvestris* sp. nov. from which it can be differentiated by a combination of these characters, but particularly in lip region shape (separated from body contour by slight depression vs anteriorly rounded continuous), and J1 tail shape (conoid vs conoid-subdigitate) (Figures 6.9, 6.10, 6.13 and 6.14). In addition, according to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (shape of anterior region), F (body length), H (tail shape) and I (presence/absence of males), *L. wicuolea* sp. nov. can be related with *L. henanus* Xu and Cheng 1992 and *L. vallensis* sp. nov. From *L. henanus* it differs mainly in having a longer body and tail length (6.1–8.7 vs 3.8–7.0 mm, 37.5–56.0 vs 24.6–42.0 µm; respectively), a shorter odontostyle length (77.0–95.0 vs 90.5–104.0 µm) and a narrower lip region width (9.5–13.5 vs 13.2–18.0 µm) (Xu and Cheng 1992, Zheng *et al.* 2001, Guo *et al.* 2011). Finally, *L. wicuolea* sp. nov. differs basically from *L. vallensis* sp. nov. by lower a and c´ ratio (79.3–115.6 vs 125.1–149.8, 0.8–1.2 vs 1.0–1.4; respectively) (Table 6.9, Figures 6.11 and 6.12).

Table 6.10 Morphometrics of females and juvenile stages of *Longidorus wicuolea* sp. nov. from the rhizosphere of cultivated and wild olives at several localities (Sevilla and Huelva provinces) southern Spain[a].

Host/locality, sample code	cultivated olive, Carmona (Sevilla province), JAO95						wild olive Bonares (Huelva, province), AR101
Characters/ratios[b]	Holotype	Paratype Females	J1	J2	J3	J4	Females
n		20	7	5	5	5	3
L (mm)	7.9	7.6 ± 0.68 (6.1-8.7)	2.06 ± 0.17 (1.86-2.25)	2.82 ± 0.34 (2.48-3.25)	3.81 ± 0.31 (3.27-4.02)	6.32 ± 1.00 (4.99-7.32)	7.5 ± 0.15 (7.4-7.7)
a	97.3	97.5 ± 9.1 (79.3-115.6)	62.8 ± 3.9 (58.0-69.0)	68.5 ± 5.3 (61.3-76.2)	82.2 ± 7.0 (71.2-88.8)	93.2 ± 4.9 (89.8-101.0)	102.6 ± 6.2 (95.6-107.0)
b	13.7	15.9 ± 2.5 (11.5-22.8)	9.1 ± 1.0 (7.8-10.4)	9.1 ± 2.2 (7.1-11.7)	11.5 ± 1.8 (9.8-14.4)	13.9 ± 2.8 (9.8-16.8)	16.3 ± 0.3 (15.9-16.6)
c	158.6	167.8 ± 14.9 (146.4-205.1)	47.5 ± 6.2 (39.7-54.6)	61.4 ± 5.8 (54.7-70.3)	79.2 ± 7.6 (71.6-91.4)	132.6 ± 35.9 (108.4-195.1)	177.3 ± 1.7 (175.9-179.1)
c´	1.0	0.9 ± 0.1 (0.8-1.2)	2.0 ± 0.2 (1.7-2.3)	1.6 ± 0.1 (1.5-1.8)	1.4 ± 0.1 (1.2-1.5)	1.0 ± 0.1 (0.8-1.1)	0.8 ± 0.1 (0.8-0.9)
V	51.0	50.6 ± 1.2 (48.0-52.5)	-	-	-	-	-
Odontostyle	86.0	86.4 ± 4.1 (77.0-94.0)	52.4 ± 1.7 (49.5-54.5)	59.6 ± 2.0 (57.0-62.0)	67.1 ± 2.4 (64.0-70.5)	79.0 ± 2.2 (75.5-81.0)	90.5 ± 6.1 (83.5-95.0)

Host/locality, sample code	cultivated olive, Carmona (Sevilla province), JAO95						wild olive Bonares (Huelva, province), AR101
Characters/ratios[b]	**Holotype**	**Paratype Females**	**J1**	**J2**	**J3**	**J4**	**Females**
Odontophore	50.0	47.8 ± 4.4 (39.5-59.0)	29.9 ± 4.4 (23.5-35.5)	38.6 ± 3.6 (34.5-44.0)	37.1 ± 3.2 (33.5-42.0)	45.0 ± 4.0 (40.5-49.0)	43.7 ± 3.8 (40.0-47.5)
Replacement odontostyle	-	-	61.0 ± 1.3 (58.5-62.0)	72.7 ± 1.3 (71.5-74.5)	79.0 ± 1.9 (77.0-81.5)	90.7 ± 4.8 (85.5-95.0)	-
Lip region diam.	10.5	10.8 ± 0.6 (9.5-12.0)	6.1 ± 0.5 (5.5-7.0)	7.3 ± 0.9 (6.5-8.5)	7.7 ± 0.7 (7.0-8.5)	9.1 ± 0.8 (8.5-10.0)	12.5 ± 0.9 (12.0-13.5)
Oral aperture-guiding ring	29.5	29.7 ± 1.6 (27.0-33.0)	20.7 ± 0.7 (19.5-21.5)	21.1 ± 1.7 (19.0-23.5)	23.9 ± 1.9 (22.0-27.0)	27.7 ± 2.0 (25.0-30.0)	26.8 ± 1.9 (25.5-29.0)
Tail length	50.0	45.6 ± 4.2 (37.5-56.0)	43.7 ± 2.8 (40.0-47.5)	46.1 ± 5.3 (41.5-55.0)	48.3 ± 4.5 (43.5-54.0)	48.8 ± 7.4 (37.5-55.5)	42.5 ± 0.9 (42.0-43.5)
J	14.5	9.3 ± 1.8 (6.0-14.5)	9.5 ± 0.9 (9.0-11.0)	6.6 ± 1.9 (5.0-8.5)	5.9 ± 1.8 (5.0-8.5)	7.1 ± 1.1 (6.0-8.5)	13.2 ± 2.5 (10.5-15.5)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).

[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; J (hyaline tail region length).

Molecular divergence of the new species:

D2–D3 sequences from *L. wicuolea* sp. nov. (KT308863-KT308866) differed with the closest related species, *L. silvestris* sp. nov. (KT308859-KT308860) by 13 nucleotides (98% similarity) and from *L. magnus* (JX445112) and *L. vineacola* (JX445110) by 60 nucleotides (92% similarity) (Table 6.5). Intraspecific variation of D2–D3 segments detected between the two studied populations of *L. wicuolea* sp. nov. consisted of 6 nucleotides (99% similarity), and no indels. Similarly, the ITS1 (KT308887-KT308889) also showed a low intraspecific variability between the two studied populations with only 4 nucleotides (99% similarity). The closet ITS1 to that of *L. wicuolea* sp. nov. was *L. silvestris* sp. nov. (KT308884) consisting in 73 nucleotides and 37 gaps (90% similarity). The partial 18S of *L. wicuolea* sp. nov. (KT308900) closely matched with several species of *Longidorus*, some of them were *L. magnus* (HM92921345, KT308902), *L. vinearum* (KT308903), *L. lusitanicus* (KT308901) and *L. silvestris* sp. nov. (KT308898).

3.2.7 Morphology and morphometrics of species of known *Longidorus* species

Morphological and morphometrical data as well as molecular delineation (rDNA) of *L. alvegus, L. intermedius*, *L. magnus*, *L. oleae* and *L. vineacola* have been previously recorded within studies of dagger and needle nematodes infesting vineyards in southern Spain [19, 28]. The new records of these species from wild and cultivated olive in Granada and Sevilla provinces presented here extend the geographical distribution of these species (Tables 6.11 and 6.12) in southern Spain (Gutiérrez-Gutiérrez *et al.* 2013). Consequently, only D2–D3 sequences had been reported here for these samples. For other known species studied, representing the first molecular characterization and new records for olive or for Spain (*viz. L. lusitanicus* and *L. vinearum*), a brief description and a morphometric comparison with previous records and paratypes is provided below (Figures 6.15 and 6.16, Tables 6.11 and 6.12).

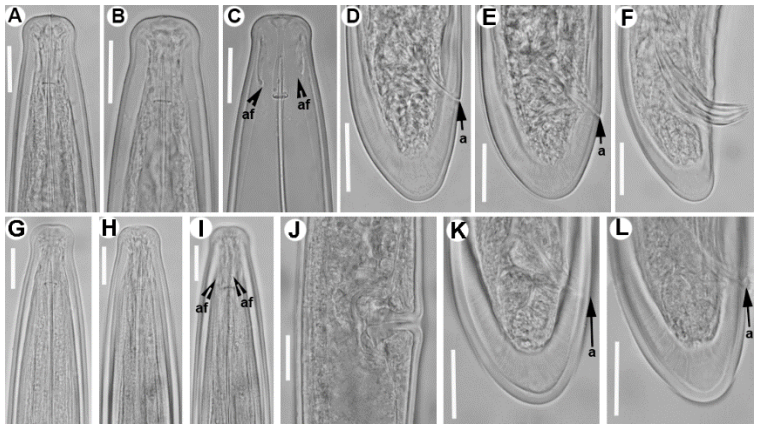

Figura 6.15: Light micrographs of *Longidorus lusitanicus* Macara 1985 from wild olive at Sanlúcar de Barrameda (Cádiz province) (A-F), and paratypes from the Nematode Collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (G-L). A-C, G-I) Female lip regions. J) Vulval region. D-E, K, L) Female tails. F) Male tail. Abbreviations: a = anus; af = amphidial fovea. (Scale bars = 20 µm).

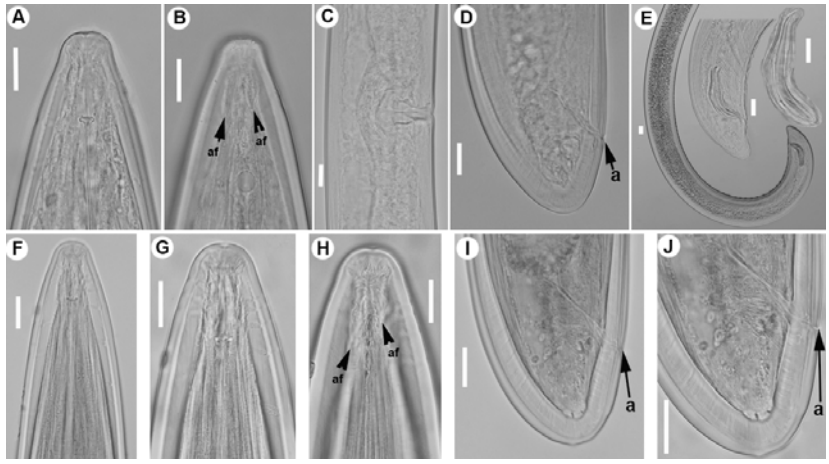

Figura 6.16: Light micrographs of Longidorus vinearum Bravo and Roca 1995 from wild olive at Santa Mª de Trassierra (Córdoba province) (A-G), and paratypes from the nematode collection at the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (H-L). A-B, H-J) Female lip regions. C) Vulval region. D, K, L) Female tails. E-G) Male tails and detail of spicules. Abbreviations: a = anus; af = amphidial fovea. (Scale bars = 20 µm).

3.2.7.1 *Longidorus lusitanicus* Macara 1985

The gonochoristic population of Longidorus from wild olive at Sanlúcar de Barrameda (Cádiz province) agrees fairly well with studied paratypes and original description of *L. lusitanicus*. This population was characterised by a lip region expanded or distinctly offset by constriction, rounded laterally and almost flattened frontally; amphidial fovea pouch-shaped, distinctly asymmetrically bilobed; female tail conoid-rounded; and the same proportion of male specimens found (Table 6.11, Figure 6.15). Morphometrics were coincident with those provided in the original description, except for only minor differences in oral aperture-guiding ring distance, which may be due to few specimens originally studied, or geographical intraspecific variability (Mendes Macara 1985). This is the first report for Spain and confirms a wider distribution in the Iberian Peninsula, apart from original description in Portugal. According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), this species has the following code: A3 B34 C23 D4 E3 F234 G2 H1 I2.

D2–D3 segments of *L. lusitanicus* (KT308869) was 95% similar to *L. vinearum* (KT308874-KT308877), *L. goodeyi* (AY601581), *L. magnus* (JX445112) and *L. onubensis* sp. nov. (KT308857-KT308858). The ITS1 of *L. lusitanicus* (KT308891) showed some homology with *L. onubensis* sp. nov. (81% similarity) and scarce homology with other ITS1 sequences from *Longidorus* species available in GenBank. The partial 18S region of *L. lusitanicus* (KT308901), was very similar to several sequences of *Longidorus* spp., including *L. vineacola* (JX445153, AY283169), *L. magnus* (HM921345) and *L. onubensis* sp. nov. (KT308897).

Table 6.11 Morphometrics of *Longidorus lusitanicus* Macara 1986 and *L. oleae* Gutiérrez-Gutiérrez *et al*. 2013 studied from southern Spain[a].

	***Longidorus lusitanicus* Macara, 1986**	
Locality/host-plant	**Bolonia (Cádiz, Spain), wild olive**	
Sample code	**J212B**	
Characters/ratios [b]	**Females**	**Males**
n	8	6
L (mm)	5.1 ± 0.52 (4.23-5.75)	5.1 ± 0.20 (4.71-5.30)
a	95.9 ± 6.1 (89.2-102.9)	104.2 ± 6.6 (95.3-112.7)
b	15.2 ± 2.0 (12.2-17.2)	17.4 ± 2.5 (13.9-21.1)
c	166.2 ± 22.6 (141.1-198.3)	148.4 ± 11.3 (130.7-165.0)
c´	0.8 ± 0.1 (0.7-0.9)	1.0 ± 0.1 (0.9-1.1)
V	50.4 ± 1.9 (47.0-54.0)	-
Odontostyle	84.8 ± 4.3 (80.0-92.0)	84.1 ± 3.5 (80.0-88.5)
Odontophore	50.6 ± 1.8 (49.0-54.0)	50.7 ± 2.7 (48.0-54.0)
Lip region diam.	21.0 ± 0.8 (19.5-22.0)	21.2 ± 0.9 (20.0-22.5)
Oral aperture-guiding ring	26.9 ± 0.8 (26.0-28.0)	27.4 ± 2.1 (24.0-30.0)
Tail length	30.9 ± 3.1 (28.0-37.0)	34.3 ± 1.7 (31.0-36.0)
Spicules	-	51.8 ± 3.2 (48.0-55.0)
Lateral accessory piece	-	14.8 ± 0.8 (14.0-16.0)

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri & Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

Table 6.12 Morphometrics of *Longidorus vinearum* Bravo and Roca 1995 populations studied from southern Spain[a].

Locality/host-plant	**Santa María de Trassierra (Córdoba, Spain), wild olive**							
Sample code	**AR059**			**AR097**		**AR066**	**AR111**	
Characters/ratios[b]	**Females**	**Male**	**J1**	**Females**	**Male**	**Female**	**Females**	**Male**
n	3	1	3	2	1	1	2	1
L (mm)	7.8 ± 0.38 (7.4-8.1)	8.1	1.7 ± 0.32 (1.4-2.0)	8.4 ± 0.13 (7.5-9.3)	8.3	7.1	7.8 ± 0.11 (6.7-8.9)	7.3
a	74.7 ± 3.0 (71.2-76.7)	70.7	46.5 ± 6.6 (39.0-51.6)	75.4 ± 11.0 (67.7-83.2)	80.8	70.3	61.6 ± 2.7 (58.8-64.2)	64.5
b	15.4 ± 3.0 (12.5-18.0)	15.3	8.0 ± 0.4 (7.6-8.3)	15.0 ± 1.6 (13.9-16.1)	15.3	13.8	14.4 ± 2.5 (11.6-16.5)	12.6
c	177.4 ± 11.8 (167.5-190.5)	147.9	33.1 ± 1.9 (31.1-34.8)	164.6 ± 18.2 (151.7-177.5)	189.0	133.4	160.4 ± 25.3 (141.0-189.1)	135.5
c´	0.6 ± 0.1 (0.6-0.7)	0.8	2.0 ± 0.26 (1.8-2.3)	0.8 ± 0.0 (0.8-0.8)	0.6	0.8	0.7 ± 0.1 (0.6-0.7)	0.9
V	46.2 ± 0.8 (45.5-47.0)	-	-	47.8 ± 0.4 (47.5-48.0)	-	49.0	48.2 ± 1.0 (47.0-49.0)	-
Odontostyle	111.0 ± 2.0 (109.0-113.0)	98.0	67.2 ± 1.4 (65.5-68.0)	105.0 ± 0.0 (105.0-105.0)	109.0	110.5	105.2 ± 4.0 (100.5-107.5)	116.5
Replacement odontostyle	-	-	71.5 ± 1.8 (70.0-73.5)	-	-	-	-	-
Odontophore	54.5 ± 2.3 (52.0-56.5)	62.5	34.5 ± 8.5 (28.5-40.5)	64.0 ± 1.4 (63.0-65.0)	68.0	72.0	75.0 ± 4.4 (72.0-80.0)	63.0

Locality/host-plant	Santa María de Trassierra (Córdoba, Spain), wild olive							
Sample code	AR059			AR097		AR066	AR111	
Characters/ratios [b]	Females	Male	J1	Females	Male	Female	Females	Male
Lip region diam.	21.5 ± 0.9 (20.5-22.0)	24.0	9.8 ± 0.8 (9.0-10.5)	21.3 ± 0.8 (20.0-22.5)	23.5	21.5	20.3 ± 1.0 (19.5-21.5)	20.5
Oral aperture-guiding ring	35.7 ± 0.8 (35.0-36.5)	38.0	21.5 ± 0.0 (21.5-21.5)	33.8 ± 0.4 (33.5-34.0)	38.5	38.0	34.8 ± 3.2 (32.5-38.5)	36.0
Tail length	44.3 ± 3.6 (42.0-48.5)	55.0	50.3 ± 6.8 (44.0-57.5)	51.0 ± 2.1 (49.5-52.5)	44.0	53.5	49.2 ± 6.0 (44.5-56.0)	54.0
Spicules	-	100.0	-	-	105.0	-	-	109.0
Lateral accessory piece	-	20.0	-	-	21.5	-	-	26.5

[a] Measurements are in µm (except for L) and in the form: mean ± standard deviation (range).
[b] Abbreviations as defined in Jairajpuri and Ahmad (1992). a, body length/maximum body width; b, body length/pharyngeal length; c, body length/tail length; c', tail length/body width at anus; V (distance from anterior end to vulva/body length) x 100; T (distance from cloacal aperture to anterior end of testis/body length) x 100; J (hyaline tail region length).

3.2.7.2 Longidorus vinearum Bravo and Roca 1995

The four gonochoristic populations of *L. vinearum* from wild olive at Santa María de Trassierra (Córdoba province) agree fairly well with studied paratypes and original description of *L. vinearum*. The four studied populations were characterised by a robust and long body, lip region anteriorly rounded and separated from body contour by a very slight depression; amphidial fovea pouch-shaped, distinctly asymmetrically bilobed; female tail short, bluntly rounded to hemispherical with rounded terminus; and the common presence of male specimens (Table 6.12, Figure 6.16). Morphometrics of female, male and J1 specimens were coincident with those provided in the original description and rather similar to data reported subsequently for other populations of Portugal, except for minor differences in a ratio and length of spicules, which may be due to few specimens originally studied or geographical intraspecific variability (Bravo and Roca 1995, 1998). This is the first report for Spain and confirms a wider distribution in the Iberian Peninsula, apart from original description and other populations in Portugal. According to the polytomous key Chen *et al*. (1997) and the supplement by Loof and Chen (1999), this species has the following code: A45 B345 C34 D2 E3 F345 G12 H1 I2.

The closet species regarding D2–D3 segments of *L. vinearum* (KT308874-KT308877) were *L. magnus* (HM921361, JX445112, 96% similarity) and *L. goodeyi* (AY601581, 94%). ITS1 (KT308892-KT308893) region also showed some similarity with *L. magnus* (HM921340, 90% similarity), but no more similarity values above 80% were found in GenBank. The partial 18S of *L. vinearum* (KT308903) matched closely (99%) with several *Longidorus* spp., such as *L. vineacola* (JX445153, AY283169) and *L. magnus* (HM921345).

3.3 Phylogenetic relationships of the *Logidorues* spp.

The amplification of D2–D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial 18S rRNA yielded a single fragment of approximately 800 bp, 1000 bp, and 1500 bp, respectively, based on gel electrophoresis.

Sequences from other species of *Longidorus* spp. obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were used for further phylogenetic studies. Sequences for *L. indalus* sp. nov., *L. lusitanicus*, *L. macrodorus* sp. nov., *L. onubensis* sp. nov., *L. silvestris* sp. nov., *L. vallensis* sp. nov., *L. vinearum*, and *L. wicuolea* sp. nov., were obtained for these species in this study. On the other hand, sequences for *L. alvegus* (KT308867), *L. intermedius* (KT308868, KT308890), *L. magnus* (KT308870), *L. oleae* (KT308871) and *L. vineacola* (KT308872, KT308873) matched well with former sequences deposited in GenBank, extending the molecular diversity of these species to the newly studied areas.

Phylogenetic relationships among Longidorus species inferred from analyses of D2–D3 expansion segments of 28S, ITS1, and the partial 18S rDNA gene sequences using BI are given in Figure 6.17, 6.18, and 6.19, respectively. To facilitate discussion, clades that were well supported or are taxonomically well founded are labelled in roman numerals from I through VII (Figure 6.17). Poorly supported lineages are not explicitly labelled. The 50% majority rule consensus 28S rRNA gene BI tree of *Longidorus* and *Paralongidorus* spp. based in a multiple edited alignment including 133 sequences and 748 total characters consisted of six moderate to highly supported major clades in the genus (Figure 6.17). Clade I is well-supported (PP = 100%) comprising 16 species including nine reported in olives: *L. vinearum* (KT308874-KT308877), *L. onubensis* sp. nov. (KT308857-KT308858), *L. silvestris* sp. nov. (KT308857-KT308860), *L. lusitanicus* (KT308869), *L. wicuolea* sp. nov. (KT308863-KT308866) and *L. macrodorus* sp. nov. (KT308855-KT308856), *L. magnus* (JX445112, HM921361, KT308870), *L. oleae* (JX445103, KT308871), *L. vineacola* (JX445110-JX445111, KT308873-KT308874) and other *Longidorus* spp. from the Mediterranean Basin such as *L. andalusicus* (JX445101-JX445102), *L. fasciatus* (JX445108), *L. iuglandis* (JX445104- JX445105), *L. crataegi* (JX445114), *L. baeticus* (JX445106- JX445107), *L. orientalis* (GU001823, KJ802877), and *L. goodeyi* (AY601581) from UK. All these species shared a hemispherical, convex-conoid and short tail. Clade II is well-supported (PP = 100%) comprising ten species and including *L. intermedius* (AY601577, JX445117, KT308868). *Longidorus vallensis* sp. nov. (KT308861-KT308862), was phylogenetically related to *L. rubi* (JX445116) forming a well-supported clade (PP = 100%), and with *L.*

alvegus (JX445115, HM921360, KT308867) which formed a sister-clade, however the BI values for this sister-clade is low. Finally, *L. indalus* sp. nov. (KT308852-KT308854) did not form supported clades with any of *Longidorus* species. Clade III is also well-supported (PP = 100%) and comprised all *Paralongidorus* species, except *P. bikanerensis* (JN032584), which clustered in the moderately supported (PP = 81%) clade IV with other species from different geographical origin. Clade V and VI are well-supported (PP = 100%) and comprised five species of Asiatic origin, and a basal well-supported (PP = 100%) clade VI with four species from different geographical origin (Figure 6.17).

Difficulties were experienced with alignment of the ITS1 sequences due to scarce homology, thus, related sequences were divided into two different groups in our study (Figure 6.18). The first group included 752 characters and 29 sequences comprising several *Longidorus* species also from the Mediterranean Basin and with hemispherical, convex-conoid and short tail, *L. lusitanicus* (KT308891), *L. macrodorus* sp. nov. (KT308880-KT308881), *L. onubensis* sp. nov. (KT308882-KT308883), *L. silvestris* sp. nov. (KT308857-KT308860), *L. wicuolea* sp. nov. (KT308884), *L. vinearum* (KT308892-KT308893), *L. vallensis* sp. nov. (KT308885-KT308886), and *L. intermedius* (KT308890) with a short body length (Figure 6.17). These results agree with those obtained for D2–D3 segments. This phylogenetic tree resolved two major well supported (PP = 100%) clades, *L. vinearum*, *L. lusitanicus*, *L. onubensis* sp. nov., *L. silvestris* sp. nov. and *L. wicuolea* sp. nov. were placed within the first major clade. *Longidorus vinearum*, *L. lusitanicus* and *L. onubensis* sp. nov. formed a high supported subclade (PP = 100%) with *L. magnus* (HM921340). *Longidorus wicuolea* sp. nov. was placed within another well supported subclade (PP = 100%) with *L. silvestris* sp. nov. and finally *L. macrodorus* sp. nov. was phylogenetically related to *L. baeticus* (JX445093). Second major clade was low-supported and was formed by five *Longidorus* species, *L. vallensis* sp. nov. was placed with *L. rubi* (JX445098) in a high-supported subclade (PP = 100%) and it was related to *L. alvegus* (HM921339) which formed another low-supported subclade. Finally, *L. intermedius* and *L. elongatus* (GU199044) formed a high-supported subclade (PP = 100%), occupying a basal position in the tree (Figure 6.18).

The second group of the ITS1 sequences included 1126 characters and 12 sequences comprising ten *Longidorus* species characterized by a

medium to short body length, including *L. indalus* sp. nov. (KT308878-KT308879), *L. profundorum* (AJ549988), *L. sturhani* (FJ009680), *L. crassus* (AF511414), *L. kuiperi* (AM905257-AM905258), *L. fragilis* (AF511418) and *L. breviannulatus* (AF511413). *Longidorus indalus* sp. nov. clustered with *L. profundorum* in a high supported clade (PP = 100%) (Figure 6.18).

The 50% majority rule BI tree of a multiple alignment including 90 18S sequences and 1687 bp and as well as in the D2–D3 and ITS1 tree, *L. lusitanicus* (KT308901), *L. macrodorus* sp. nov. (KT308896), *L. onubensis* sp. nov. (KT308897), *L. silvestris* sp. nov. (KT308898), *L. vinearum* (KT308903) and *L. wicuolea* sp. nov. (KT308900) clustered within the same well-supported (PP = 100%) clade with *Longidorus* species from Mediterranean Basin and sharing a convex-conoid female tail shape such as *L. andalusicus* (JX445118), *L. oleae* (JX445119), *L. vineacola* (JX445123, AY283169), *L. magnus* (HM921345-KT308902), *L. baeticus* (JX445121), *L. fasciatus* (JX445122) and *L. iuglandis* (JX445120). Phylogenetic inferences based on 18S also suggest that *L. vallensis* sp. nov. and *L. rubi* are close-related species (PP = 100%). Finally, *L. indalus* sp. nov. (KT308894-KT308895) clustered in this case with *L. dunensis* (AY284819) with a low support (PP = 81%).

Figura 6.17: The 50% majority rule consensus tree from Bayesian inference analysis generated from the D2–D3 of 28S rRNA gene dataset of *Longidorus* spp. with the SYM+I+G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site. A). Clades I & II. B). Clades III-VI.

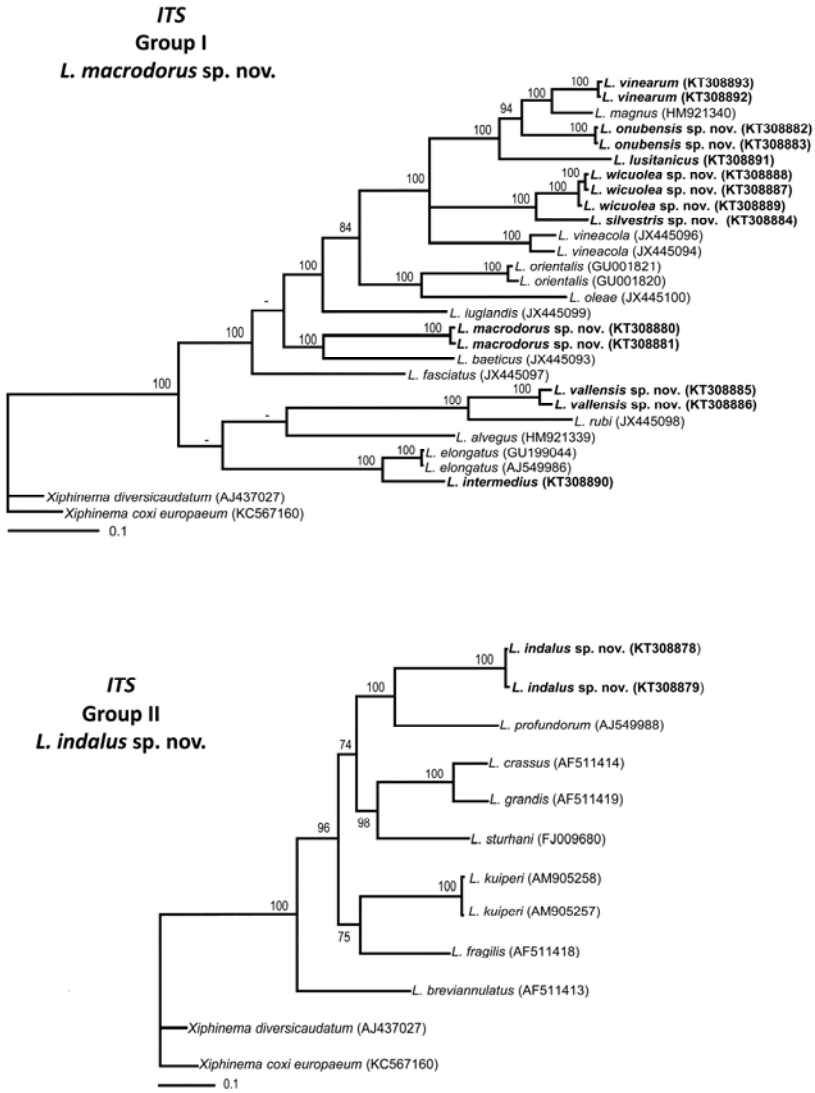

Figura 6.18: The 50% majority rule consensus trees from Bayesian inference analysis generated from the ITS rRNA gene dataset of *Longidorus macrodorus* sp. nov. group and *L. indalus* sp. nov. group with the TVM+I+G and TIM3+I+G models, respectively. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site.

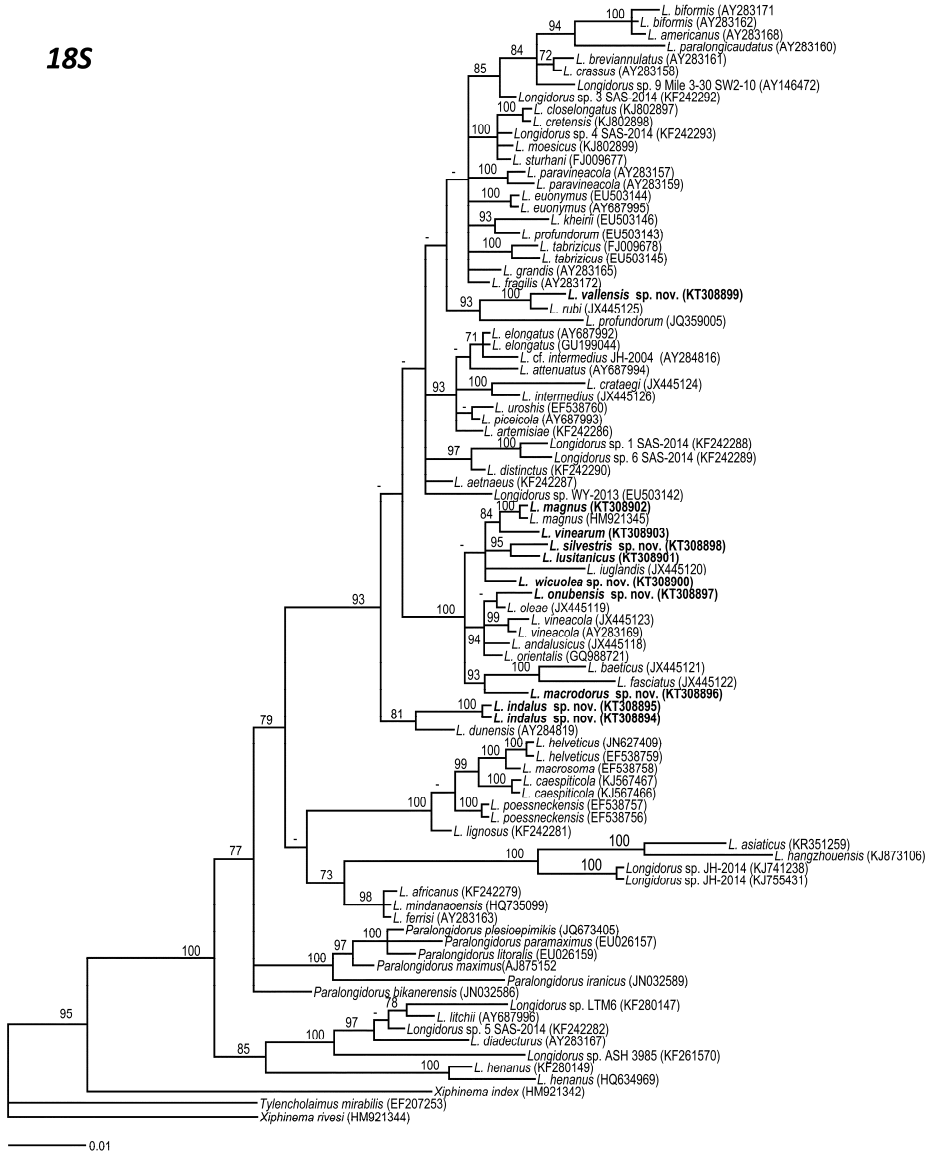

Figura 6.19: The 50% majority rule consensus trees from Bayesian inference analysis generated from the partial 18S rRNA gene dataset of *Longidorus* spp. with the TVMef+I+G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold letters. Scale bar = expected changes per site.

4. Discussion

The primary objective of this study was to unravel the biodiversity, distribution and molecular phylogeny of needle nematodes of the genus Longidorus associated with wild and cultivated olives in southern Spain. This was conducted in an extensive and systematic nematological survey that included 159 locations and 449 sampling sites. We found 40 Spanish populations of *Longidorus* spp. infesting olive soils. Our results demonstrate that the use of morphological studies together with rDNA molecular markers may decipher the specific biodiversity in this complex group of plant-parasitic nematodes. We described here six new Longidorus species, based on integrative taxonomy and the phylogenetic relationships of the genus *Longidorus* based on nuclear rDNA markers.

The comparative morphological and morphometrical study of the 40 Spanish populations of *Longidorus* spp. confirmed that diagnosis and identification of these species based solely on diagnostic morphometric features is quite complex since there is almost a continuous range of character measurements within populations as well as among species (Gutiérrez-Gutiérrez *et al.* 2013, Palomares-Rius *et al.* 2014). The present results (including new and known species) enlarge the biodiversity of *Longidorus* in the Iberian Peninsula and agree with previous data obtained for the phylogeny and biogeography of the genus *Longidorus* in the Euro-Mediterranean region (Navas *et al.* 1990, 1993, Gutiérrez-Gutiérrez *et al.* 2011, 2013), in which a dispersalist model was one of the primary explanations for the large groups of Longidorus species found in this region.

Considering the species richness of PPN associated with olive in different studies, the genus Longidorus is one of the most biodiverse with nine species (*viz. L. africanus*, *L. belloi*, *L. closelongatus*, *L. cretensis*, L. elongatus, L. macrosoma, L. oleae, L. pseudoelongatus, L. siddiqii, and *L. vinearum*) reported in several countries of the Mediterranean Basin such as Egypt, Greece, Jordan, Portugal and Spain (Tarjan 1964, Hashim 1979,

Peña-Santiago 1990, Lamberti *et al.* 1996, Bravo and Roca 1998, Tzortzakakis *et al.* 2008, Castillo *et al.* 2010, Ibrahim *et al.* 2010, Gutiérrez-Gutiérrez *et al.* 2013, Ali *et al.* 2014, Tzortzakakis *et al.* 2014). Although all *Longidorus* spp. are obligate soil plant ecto-parasites of a wide range of wild and cultivated plants causing enlarged swellings of root tips, it is unlikely that these species could be detected in other wild and cultivated plants in the next future. The present results double the previous biodiversity of Longidorus species detected in olive worldwide, including six new species and two new records for wild and cultivated olives (*L. alvegus* and *L. vineacola*), as well as two additional new records for wild olives (*L. intermedius* and *L. lusitanicus*). The most recent major geological event having important effects for nematode biodiversity and distribution in Europe was the Quaternary glaciation which happened *ca.* 40,000 years ago. In Europe has been hypothesized that reduced species numbers in northern Europe is attributed to Quaternary glaciations, being the highly diverse nematofauna of the Mediterranean basin related to Miocene plate tectonics in that area (Topham and Alphey 1985). Our study showed a great diversity in Southern Spain. However, because of no sampling North-South has been developed; more intensive studies are needed in northern areas in order to corroborate this hypothesis. The distribution of the 40 Longidorus populations collected in Andalusia showed that some of them revealed a certain geographic association to western areas (*viz. L. alvegus*, *L. intermedius*, *L. lusitanicus*, *L. onubensis* sp. nov., *L. vineacola*, *L. vinearum*, *L. wicuolea* sp. nov.) and eastern regions (*viz. L. indalus* sp. nov.), while only *L. magnus* was detected in both areas (Figure 6.1). The present findings showed certain coincidences with the quantitative analysis of *Longidorus* spp. distribution carried out by Navas *et al*. (1990), who recognized two main groups of species, the European-Atlantic and the Mediterranean. The widespread distribution of *L. magnus* may suggest a high ecological flexibility e.g. adaptability to a range of soil types, and reproduction sustained over a broad temperature range (Brown *et al.* 1994). While other species seems to be better adapted to drier areas as it is the case for *L. indalus* sp. nov. in Eastern regions with markedly lower precipitation. Species showing a restricted distribution may be the result of isolation of populations in diverse biotopes which would result in reproductive isolation and hence the establishment of new species (Brown *et al.* 1994). Also, although agricultural activities may result in the widespread dissemination of *Longidorus* species (Brown *et al.* 1994), the geographical distribution of *Longidorus* species in wild and cultivated olives

in southern Spain suggest an established pattern related to ecological factors, on a geological timescale. These nematodes could have a lower dissemination by human activities than other plant-parasitic nematodes (i.e. cyst- or root-lesion nematodes) because of their sensitivity to fast desiccation, large body size, and the absence of survival-resistance forms. Unfortunately, little is known about the ecological requirements of *Longidorus* nematodes and elucidation of speciation and species biodiversity has currently to be approached on the groupings of morphometric characters (Brown *et al.* 1994). Consequently, further research is needed in order to determine the influence of physico-chemical soil factors on the incidence and distribution of these nematodes in southern Spain and other wider areas.

Sequences of nuclear ribosomal RNA genes, particularly D2–D3 and ITS1, have proven to be a powerful tool for providing accurate species identification of Longidoridae (He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2012, Palomares-Rius *et al.* 2013). However, D2–D3 expansion region was more useful for establishing phylogenetic relationships among *Longidorus* species than ITS1 or 18S. The great diversity detected in the ITS1 suggests that a variety of poorly understood factors are involved in the fast evolution of this region in nematodes. Thus, ITS1 appears better suited for differentiation of species than for phylogenetic relationships within *Longidorus*. Our findings also confirm that partial 18S sequence does not have enough resolution to distinguish species, because different species showed a low nucleotide differences amongst them. Phylogenetic analyses based on D2–D3, ITS1, and partial 18S using BI resulted in a congruent position for the newly sequenced species of *Longidorus* spp. from Spain, which grouped in a separate clade, except for *L. vallensis* sp. nov. (KT308861-KT308862) and *L. indalus* sp. nov. (KT308852-KT308854) in the D2–D3, partial 18S, and ITS1 trees, which grouped separately (Figures 6.17, 6.18 and 6.19). *Longidorus vallensis* sp. nov. clustered in all ribosomal markers with *L. rubi*. However, these species showed several morphological differences that made it difficult to establish a correspondence between morphological characters and the phylogenetic trees inferred from the molecular data. The majority of the species showed congruence in the phylogenetic relationships within these ribosomal markers using the DNA from the same individual. However, *L. indalus* sp.

nov. phylogenetic position was not congruent amongst the different ribosomal markers used here. This could be a result of different mutation rates within the different ribosomal markers, or difficulties in sequence alignment in ITS1 sequences. The phylogenetic relationships inferred in this study based on the D2–D3 and ITS1 sequences mostly agree with the lineages obtained by other authors (He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2011, 2013, Amrei *et al.* 2013, Subbotin *et al.* 2014). Most of the newly and known described species in this research (viz. L. lusitanicus, *L. macrodorus* sp. nov., *L. magnus*, *L. oleae*, *L. onubensis*, *L. silvestris* sp. nov., L. vineacola, *L. vinearum*, *L. wicuolea* sp. nov.) grouped genetically in the same clade. These species shared a long body and odontostyle and can be considered as the most evolved species in the genus (Coomans 1996). These traits could be related to the feeding habits of these nematodes, since longer stylets are better adapted to penetrate major woody plants roots persisting during the hot-dry summer conditions prevalent in Southern Spain and with long body sizes to move quickly deeper in the soil to avoid dry conditions in summer.

To confirm the correlation of the results obtained by conventional morphological approaches and new molecular methods is important for the proper understanding of the evolution of the genus *Longidorus*. The close relationship of the morpho-species groups detected in this and previous studies in Spain was also supported by molecular data (most of the species described were in the same clade), an observation that points to the Iberian Peninsula as a possible center of recent speciation (Gutiérrez-Gutiérrez *et al.* 2013), as it was suggested for other genera such as *Xiphinema* (Coomans *et al.* 2001, Gutiérrez-Gutiérrez *et al.* 2011, 2012, 2013), *Trichodorus* (Decraemer *et al.* 2013) or *Rotylenchus* species (Cantalapiedra-Navarrete *et al.* 2013).

5. Conclusions

In summary, the present study establishes the importance of using integrative taxonomic identification highlighting the difficulty of a correct identification at species level within the genus *Longidorus*. This study also provides molecular markers for precise and unequivocal diagnosis of some species of *Longidorus* in order to differentiate virus vector or quarantine species. This and previous studies demonstrate that the genus *Longidorus* is clearly a complex group and much work remains to be done to elucidate species boundaries in this economically important group of PPN. Furthermore, similar intensive and extensive integrative studies on *Longidorus* species in several wider areas may help to elucidate if *Longidorus* species have originated in Southeast Africa and India, when these two areas were still united, and a later spread to Laurasia was followed by a main speciation of Longidorus in the Holarctic region, especially Europe, as hypothesised by Coomans (1996). This hypothesis is reinforced with the basal position of Asian species in D2–D3 region and partial 18S phylogenetic trees.

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7. Author Contributions

Conceived and designed the experiments: PC AAY JANC JEPR CCN. Performed the experiments: PC AAY JANC JEPR CCN. Analyzed the data: PC AAY JANC JEPR CCN. Contributed reagents/materials/analysis tools: AAY JEPR CCN. Wrote the paper: PC AAY JANC JEPR CCN.

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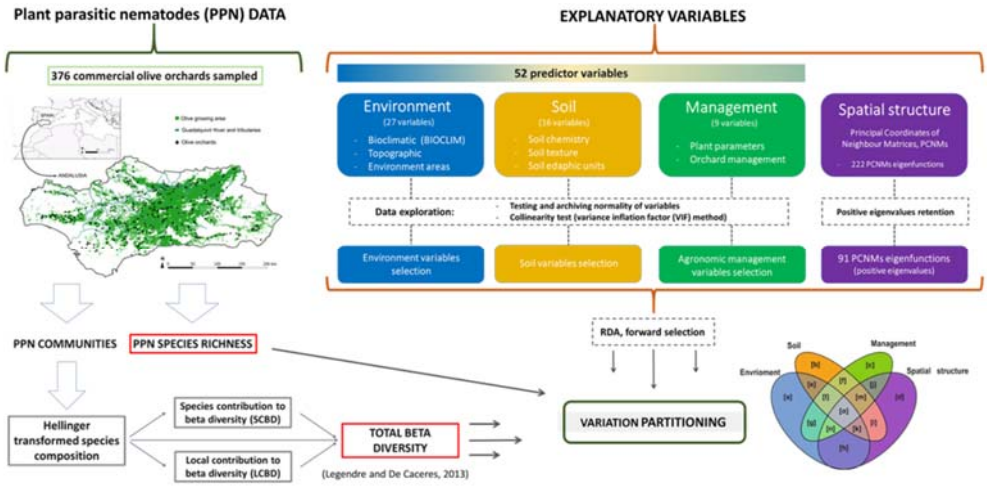
4 BLOQUE III

DESCIFRANDO LA DIVERSIDAD BETA DE NEMATODOS FITOPARÁSITOS ASOCIADOS AL CULTIVO DEL OLIVO



C6

Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olives in southern Spain

Antonio Archidona-Yuste, Thorsten Wiegand, Pablo Castillo, Juan A. Navas-Cortés

***Agriculture, Environment and Ecosystem,* en revisión**

Abstract

Numerous studies have documented spatial structures in the distribution of plant and animal communities, but relatively little is known how the diversity of soil organisms such as plant-parasitic nematodes (PPN) is structured. Host plants, such olives, are of special interest because they host a large number of PPN and have a high economic and cultural importance. In this study, we investigated how different sets of variables describing the environment (i.e. soil, above-ground environment, and agricultural management) and spatial structure influenced the variation of community composition and species richness of PPN infesting the soil rhizosphere in 376 commercial olive orchards in southern Spain. To this end, we used variation partitioning to assess the relative and shared contributions of the different groups of variables. In order to identify sites and species of particular interest, we partitioned beta diversity into local and species contributions. Contrary to the expectations that soil and management would largely determine PPN community structure, we found that more than two-thirds of the variation remained unexplained. Space and soil variables were the most important effect on both species richness and beta diversity. However, effects of agronomic practices on species richness were lower than expected, whereas they had no effect on beta diversity. We found relatively high levels of shared contributions of the different sets of variables, especially with space, indicating spatial gradients in the environmental variables. Species contributions to beta diversity (SCBD) were positively correlated with nematode prevalence and density range, suggesting that SCBD could be related with niche position as reported in other ecosystems. Our findings reveal novel insights in the spatial structuring of PPN diversity and show that their beta diversity is less structured by space and environmental factors as compared to other organism types. Overall, we reveal new insights about the pure influence of environment (e.g. above-ground environment and soil) in comparison to agricultural management (e.g. irrigation regime, cultivars, cover vegetation, etc.) on patterns diversity of PPN in agricultural ecosystems. In this regard, agricultural management shows no effect on the variation of nematode community composition in contrast to species richness, which could indicate that some species of PPN could be influenced by the use of different agronomic practices.

ADDITIONAL KEYWORDS: below-ground ecosystems – soil nematodes – ecological uniqueness – beta diversity – variation partitioning – olive

1. Introduction

The diversity of soil organisms is huge, with thousands of species often occurring within a single square meter, and a single gram of soil can contain a diverse range of nematodes, protozoa, earthworms, mites, molluscs, arthropods, and arachnids among other soil fauna (Bardgett 2005). However, while ecologists have long been fascinated by the vast diversity of organisms in terrestrial environments and by the processes that drive their diversity in space and time (Bardgett and van der Putten 2014), relatively little efforts have been dedicated to unravel below-ground agroecosystems processes that may influence the spatial structure of this diversity.

Nematodes are the most diverse Metazoan taxa on Earth with approximately one million species (Bardgett and van der Putten 2014). They are the most common and diverse multicellular terrestrial animals, being found in all soil environments (Ferris *et al.* 2001). In addition, nematodes occupy all consumer trophic levels within the soil food web, which allows them to play a central role in numerous soils functions, such as transferring energy between ecological networks (Ferris *et al.* 2001). Nematodes are also frequently associated with other organisms including plants, fungi, bacteria, micro-arthropods and other nematodes, or attack of them as parasites. Over 4,000 nematodes species have been identified as plant feeding or plant-parasitic nematodes (PPN) (Gaugler and Bilgrami 2004).

Although 15% of the nematode species richness is identified as PPN, only a small group of them are of economic importance by causing direct damage or acting as virus vector (Nicol *et al.* 2011). An important example for this is the olive tree (*Olea europaea* L.), both in wild and cultivated forms, that serves as a host to a wide diversity of PPN, including both endoparasitic and ectoparasitic species (Castillo *et al.* 2010, Ali *et al.* 2014). Recent studies have reported an exceptional diversity and prevalence of ectoparasitic PPN belonging to the Longidoridae family that infests soils from the rhizosphere of olive trees (Archidona-Yuste *et al.* 2016a, b). Soils infested by PPN can reduce growth of the cultivated olive

(*Olea europaea* subsp. *europaea* var. europaea) and they are responsible for yield losses of 5 to 10% (Castillo *et al.* 2010).

Although considerable work has been performed on nematode ecology, relatively little attention has been paid to investigate the ecological factors that control the spatial variation in species richness and community composition of PPN. Traditionally, the host plants are considered as the most important driver of PPN populations (Norton 1989, Neher 2010). Additionally, soil abiotic factors drive the distribution of PPN in both, natural (Freckman and Virginia 1989) and agriculture ecosystems (Duyck *et al.* 2012), but studies investigating the effect of multiple gradients in the above-ground environment (e.g. climate and topographic variables) as drivers of PPN patterns at regional scale produced contrasting results that make it difficult to generalize (Duyck *et al.* 2012, Palomares-Rius *et al.* 2015). While "pure" spatial structure, *i.e.* pure that comprise spatial component independent of any measurable environmental variables (Legendre *et al.* 2009), caused by limited dispersal (that acts independently of spatially structured environmental drivers) is an important factor driving beta diversity in plant communities (Hubbell 2001, De Cáceres *et al.* 2012, Baldeck *et al.* 2013a) and other major organism types (Soininen 2016). However, it is not clear if such effects occur in PPN. What´s more, PPN may be spatially distributed through movement of farm machinery, seeds, and animals or via water currents and air movement (Castillo *et al.* 2010, Neher 2010). Additionally, agronomic practices can reduce the diversity of the nematode community and use of herbicide under the tree canopy can decrease soil nematode structure and abundance in comparison to areas not treated (Sánchez-Moreno *et al.* 2009). In plant communities, a considerable proportion of variation in species composition (and species richness) remained unexplained by variables describing pure spatial structure and the environment (e.g., Baldeck *et al.* 2013b). It can therefore be expected that a similar result may hold for PPN, although deterministic effects of soil and agricultural management may strongly determine PPN community structure (Neher 2010).

We ask here about the role of environmental factors (e.g. soil, above-ground environment, and agricultural management), spatial structure (i.e. any non-random spatial organization in the distribution of communities

Peres-Neto and Legendre 2010), and stochasticity in controlling the variation in species richness and species composition of PPN communities among sites. To this end, we use variation partitioning (Borcard *et al.* 1992, Legendre and Legendre 2012) to assess the relative and shared contributions of the different environmental factors and spatial structure on the variation of PPN species richness and community composition. In the simplest case, with only one set of environmental variables, this method allows partitioning the variation among sites into four complementary components: (a) "pure environment" (non-spatial environmental factors), (b) "spatially structured environment" (induced by spatially structured environmental variables), (c) "pure space" (spatial autocorrelation independent of environment variables), and (d) "undetermined" (either due to stochasticity or caused by omission of explanatory variables) (Legendre *et al.* 2009). We extend this approach to consider the contribution of several sets of environmental variables that are hypothesized to determine the spatial variation in species richness and community composition of PPN.

Variation partitioning allows the identification of common and unique contributions of different environmental predictors as well as endogenous and exogenous spatial autocorrelation (Anderson *et al.* 2011). In addition, the variation of species composition among sites (i.e., beta diversity; Whittaker 1960, Anderson *et al.* 2011), can be partitioned into the contribution of single sites (LCBD; ecological uniqueness of sites in terms of community composition) or into the contribution of individual species (SCBD: the relative importance of each species in affecting beta diversity) (Legendre and De Cáceres 2013). Patterns in LCBD could be influenced by environmental conditions and/or general characteristics of the PPN community (e.g. community richness and abundance (Heino and Grönroos 2017) and significant LCBD values may therefore point to sites with exceptional species composition (e.g., with rare species combinations), to degraded sites, or to sites with particular ecological conditions (Legendre and Gauthier 2014). In turn, SCBD may point to species of particular importance for beta diversity and could be associated with general species characteristics (e.g., occupancy, abundance, niche) (Heino and Grönroos 2017).

More specifically, our research aims were to assess the relative contribution of environment, soil, management agricultural practices and

spatial structure in explaining the variation of community composition and species richness of PPN of the cultivated olive in southern Spain. We also assessed local and species contributions to identify their particular importance for beta diversity (LCBD and SCBD, respectively) and its drivers. To accomplish our objectives, we compiled a wide range of explanatory variables and grouped them into four sets of previously mentioned predictors. These variables represent the main hypotheses on the mechanism driving the spatial variation in species composition and species richness of PPN. The set of environmental variables included climate and topographic variables; the set of soil data variables included physicochemical, texture and edaphic properties; and the set of agronomic management variables were represented by plant parameters and the orchard management system. Finally, the set of spatial descriptors were derived by the method of principal coordinates of neighbour matrices (PCNM) (Borcard and Legendre 2002) that create spatial descriptors from the spectral decomposition of spatial relationships between the sampling sites (Borcard *et al.* 2004). Beta diversity was computed as the total variance of the transformed abundance-biomass community data to PPN species level (Legendre *et al.* 2005).

We selected the olive growing area of southern Spain as study area because of its high agriculture and socio-economic importance and because of the extensive distribution of cultivated olives (Infante-Amate 2012, MAGRAMA 2016). Additionally, it includes a wide range of ecological gradients including climate, soil, and topographic components (Ortega *et al.* 2016), as well as the large variety of agronomic management practices covering the diversity of cropping systems (e.g. from traditional to high-density orchards) (REDIAM 2016). We analyzed here data from 376 commercial olive orchards.

2. Material and Methods

2.1 Study area, soil-sampling design

The study was conducted in Andalusia, southern Spain, covering an area of approximately 90,000 Km^2 (extent: 35.9377 to 38.7289; -1.6272 to -7.5226) (Figure 7.1). Andalusia is a geomorphological heterogeneous area characterized by high mountain ranges surrounded by extensive lowlands of alluvial origin with elevation ranging between 0 and 3,479 m a.s.l. (Sierra Nevada). The south of Spain is characterized by Mediterranean climate, but with a strong influence of the Atlantic Ocean. Mean annual temperatures range from 10 to 20ºC, and mean annual rainfalls range mostly from 350 to 1,000 mm showing strong seasonality (REDIAM 2016).

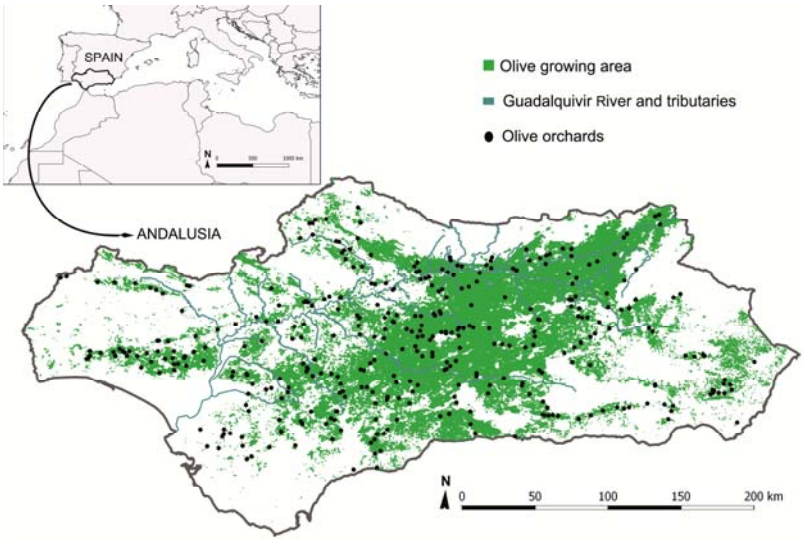


Figura 7.1: Map of Andalusia (southern Spain) showing the olive growing area including the location of the commercial olive orchards sampled (376 sampling sites) and the Guadalquivir River and tributaries.

Olive cultivations cover in Andalusia more than 1.6 million ha and account for 19% of the total surface area of the region (MAGRAMA 2016). Based on a classification of olive growing areas into biological zones (REDIAM 2016), a total of 376 commercial olive orchards were selected for

this study (Figure 7.1). Permission for sampling the commercial olive orchards was granted by the landowner.

Soil samples were collected from 2011 to 2016 during the spring season, when soil environmental conditions are favorable for biological nematode activity (Norton 1978) and guarantee an homogeneous sampling through time. In each commercial olive orchard, soil samples were taken from four to five trees that were georeferenced. Soil samples were collected with a hoe discarding the upper 5-cm top soil profile, from a 5- to 50-cm depth, in the close vicinity of active olive roots. We ensured that roots from other plants were not included. Finally, all individual samples were thoroughly mixed to obtain a single representative sample for each commercial olive orchard before nematode extraction and physicochemical parameters determination. Nematodes were extracted separately from two 250-cm^3 subsamples using magnesium sulphate centrifugal-flotation method (Coolen 1979, Castillo *et al.* 2010). The two 250 cm^3 of soil subsamples were mixed in a single one in order to carry out the diagnostic and identification of nematodes from a 500 cm^3 of soil subsample. For estimation of beta diversity, we derived for each species present at a given orchard its relative total biomass that takes into account the abundance and nematode size (which varies greatly among species). For each commercial olive orchard, nematode abundance was calculated as the total number of PPN per 500 cm^3 of soil. Relative nematode individual fresh biomass was calculated according to an adjusted Andrassy´s formula (Andrássy 1956), wherein relative biomass (µg) = $L \times D^2 * 1{,}600{,}000^{-1}$; where L is nematode body length (in µm), and D is nematode maximum body width (in µm). The nematode size was obtained using the "Nematode-Plant Expert Information System" (Nemaplex; http://nemaplex.ucdavis.edu/) and original descriptions of nematode species. Additionally, we determined for each orchard the number of nematode species found.

2.2 Explanatory variables

Variables representing habitat heterogeneity were compiled into four sets related to the environment, soil, agronomic management and spatial patterns. The environmental data set comprised 27 broad range abiotic

variables including bioclimatic predictors (BIOCLIM) based on temperature and precipitation (Nix 1986), explanatory variables related with topography such as aspect and slope, global solar radiation (GR), annual average olive potential evapotranspiration (PET), a standardized drought index (DI), and a categorical variable related with phytoclimatic areas (CA).

The second set of variables comprised soil physicochemical parameters, texture and soil edaphic type. Explanatory variables related to soil physicochemical parameters comprised twelve parameters including cation exchange capacity (CEC), Ca, Mg, exchangeable K, Na, carbonate content (CO3), extractable P, soil organic matter (SOM), total organic carbon (Corg) and nitrogen (Norg), C:N ratio, and pH (KCl).

A third set of variables included agronomic management factors that were related either to plant parameters or to orchard management. For the first subset, we used age of olive plantation and olive cultivars. The second subset comprised seven categorical variables (e.g., olive plant density, irrigation regimen and system, agronomic practices below olive tree canopy and on alley of the olive orchard, and type of vegetation cover on alley of the olive orchard).

Finally, we included explanatory variables related to spatial patterns. Spatial predictors were computed across the 376 commercial olive orchard points using principal coordinates of neighbour matrices (PCNM) (Borcard and Legendre 2002, Borcard *et al.* 2004, Dray *et al.* 2006). The spatial PCNM eigenvectors were obtained by the eigenvalue decomposition of a principal coordinate analysis (PCoA) of geographic connectivity matrices among sampling sites, resulting in orthogonal spatial patterns with higher and lower eigenvalues corresponding to broader- and finer-scale, respectively. Following Borcard and Legendre (2002), a total of 222 PCNM eigenfunctions were generated, and 91 PCNMs with positive eigenvalues were retained. The PCNM analysis was carried out using the pcnm function implemented in the package vegan (Oksanen *et al.* 2017) as described by Legendre *et al*. (2009).

2.3 Statistical analyses

Data analyses included four steps: calculation of beta-diversity, selection of explanatory variables, variation partitioning, and determination of the

contribution of sites (LCBD) and species (SCBD) to beta diversity, as well as their relationships with explanatory variables and prevalence of PPN. All statistical analyses were performed using the R v. 3.3.0 freeware (R Core Team 2016).

In a first step, prior to any statistical analysis, the biomass - site matrix data of the PPN were transformed using a Hellinger transformation (Rao 1995) that is recommended when the data matrix contain zeros, extreme values and double absences of species per site (Legendre and Gallagher 2001, Legendre and De Cáceres 2013) as occurs in our study.

2.3.1 Beta diversity computation

Total beta diversity was estimated as the total variance of the transformed abundance-biomass community data table as described by Legendre *et al*. (2005). This approach allows partitioning total beta diversity into the sum of the contributions of species (SCBD) and local sampling sites (LCBD) (Legendre and De Cáceres 2013). LCBD values, which sum to 1, were tested for significance by random distribution of species among sampling sites as null hypothesis (using 999 random permutations and preserving species abundance data) (Legendre and De Cáceres 2013). We also mapped the spatial variation of LCBD values among sampling sites. Beta diversity and the derived indices were computed using the beta.div function implemented in the package adespatial (Dray *et al.* 2017).

Finally, species richness and community composition maps were produced to summarize the spatial variation in the study area (Baldeck *et al.* 2013b). For the beta diversity map, this approach conducts non-metric multidimensional scaling (NMDS) ordination analysis on the biomass-abundance matrix data using the Bray-Curtis dissimilarity index with three dimensions and 100 random stars as arguments to obtain a low value of stress statistic index. Then, scores from the three axes were translated to a RGB colour as described by several authors (Thessler *et al.* 2005, Baldeck *et al.* 2013b). Sampling sites with more similar species composition were represented by more similar colour patterns, which were then interpreted by prevalence level of PPN (rare and common species) regarding to species

scores ordination axes from the NMDS analysis. The NMDS ordination analysis was computed using the metaMDS function found in the package vegan (Oksanen *et al.* 2017).

2.3.2 Selection of explanatory variables

Prior to statistical analyses, data transformation and normalization was carried out in each set of variables. All numeric variables were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner test). Then, in order to archive normality of variable, slope and altitude variables (environment set) were transformed by arcsine and square root, respectively; while $\log_{10}(x + 1)$ transformation was used for plant age (agronomic management set). Likewise, in order to remove any unit effects, all variables corresponding to soil chemical data from the soil set were standardized. Additionally, predictor variables were tested for collinearity (Zuur *et al.* 2010). To minimize collinearity effects we used the variance inflation factor (VIF) method that iteratively excludes numeric covariates within each variable set that showed VIF values > 3 (Zuur *et al.* 2010).

In a second step, a forward selection procedure was performed to keep for each set of explanatory variables only those variables that were significantly correlated with the species richness (or the community composition) data. For this step, categorical variables were transformed as dummy variables. We used a modified forward selection method (Blanchet *et al.* 2008) based on a permutation procedure with two stopping criteria (using 9999 random permutations); in the first step it adds variables to selection set until exceeding the critical p value (alpha threshold = 0.05), and the second step was based to the final model adjusted R^2 value to not exceed that of the global model, carried out using the package packfor (Dray 2011).

2.3.3 Variation partitioning

Variation partitioning (Borcard *et al.* 1992, Peres-Neto *et al.* 2006) was based on partial redundancy analysis for community composition of PPN (beta diversity) and on partial multiple linear regression for species richness. This allowed us to quantify the relative contribution (pure and joint

fractions) of the four sets of explanatory variables to the total variation of beta diversity and species (Borcard *et al.* 1992, Peres-Neto *et al.* 2006). To facilitate the comparison of our results, we standardized the variation explained by pure and joint fractions with the total variation explained. Variation partitioning analysis was performed using the varpart function implemented in the package vegan (Oksanen *et al.* 2017).

2.3.4 LCBD and SCBD explanatory variables

Following Heino and Grönroos (2017), we used a combination of multivariate methods to examine the variation in the local site contribution to beta diversity (LCBD) or species contribution to beta diversity. To determine variables that correlate with LCBD and SCBD we carried out multiple regression models with both community and ecological metrics as predictors. For the relationship between LCBD (and SCBD) and explanatory variables from each sampling site we used a partial linear regression according to Legendre and Gauthier (2014).

To estimate the amount of variation of the dependent variable that can be assigned solely to one set of predictors, having been taken into account the effect of the other factor, we used partial linear regression (Legendre and Legendre 2012). We performed this analysis by controlling the effects of the spatial component of LCBD by using the principal coordinates of neighbour matrices as variables, thereby determining the effect that can be exclusively attributed to the ecological variables at each sampling site. We used all explanatory variables included in the environment, soil and agronomic management sets together as ecological predictors. Then, we selected the most influent variables determining the LCBD patterns by forward selection using the same criteria as described above (double stopping and 9999 random permutations) (Blanchet *et al.* 2008).

3. Results

3.1 Beta diversity of PPN

A total of 128 PPN species from 13 families were identified in the soil samples of the 376 commercial olive orchards. The total number of PPN in each olive orchard ranged from seven (site O031) to 19,796 (site O333) nematode specimens per 500 cm^3 of soil, and the species prevalence ranged from 0.3 (several nematodes species detected only in one sampling site) to 72.6% (*Merlinius brevidens*). Migratory ectoparasite PPN such as *Helicotylenchus oleae* and *Ogma rhombosquamatum* showed the highest abundance (19,720 and 9,800 nematodes per 500-cm3 of soil, respectively). However, rare (i.e. low prevalence) sedentary endoparasitic PPN species such as *Meloidogyne javanica* were also detected at a high abundance (i.e. 10,000 nematodes per 500-cm^3 of soil).

Overall, species richness showed a wide variation among the 376 sampling sites (Figure 7.2a). This resulted in a remarkable variation in community patterns, as visualized by the beta diversity map (Figure 7.2b). The grey sites in this map comprise generally common PPN species (high prevalence) such as migratory ectoparasitic PPN, whereas sites with prevalence of rare species were visualized by the radial gradient thorough the RGB colour spectrum (Figure 7.2b).

3.2 Selection of explanatory variables

Overall, the total number of variables significantly affecting variation of PPN community patterns was higher for beta diversity (17) than species richness (10). Approximately one quarter of the variation in beta diversity was explained by the four sets of variables, with soil and spatial variables explaining the majority and accounting for 8% each (Supplementary Information). In contrast, approximately 58% of the variation in species richness was explained by the four sets of variables, with spatial variables accounting for 26% and soil for 13% (Supplementary Information).

Thirteen PCMN spatial variables with a positive eigenvalue were chosen for community composition and species richness to represent spatial structure. Soil physicochemical variables were the most influential soil variables for beta diversity and variation in species richness, followed by soil texture and edaphic type. Beta diversity was also affected by chemichal parameters such as CEC, pH (KCl), Na, Mg (Supplemantariy

Information) whereas variation in species richness was affected by only the two physicochemical parameters Na and extractable P. Soil extractable P accounted for 7% of the variation in species richness (Supplemantary Information), which indicates that this factor could play a key role on community patterns of PPN infesting soils from cultivated olive. Soil texture and edaphic type were also important but with a lower effect than physiochemical variables (Table 7.1, Supplementary Information).

Interestingly, variables considered within the environment data set explained only a low proportion of beta diversity (5%) and species richness (7%), with variables closely related to climate being the most important ones (Table 7.1, Supplementary Information). The most important predictor for both metrics was the annual precipitation (BIO12) and isothermality (BIO3), and variation of species richness was related with rainfall deficit factor (RD).

Agronomic management accounted 11% to explain of the variation in species richness, but only by 5% to beta diversity (Table 7.1, Supplementary Information). Only four management factors accounted for that variation, with irrigated class being the most important one. The age of olive trees and soil-vegetation cover affected both species richness and beta diversity, while the olive tree cultivar influenced only the latter.

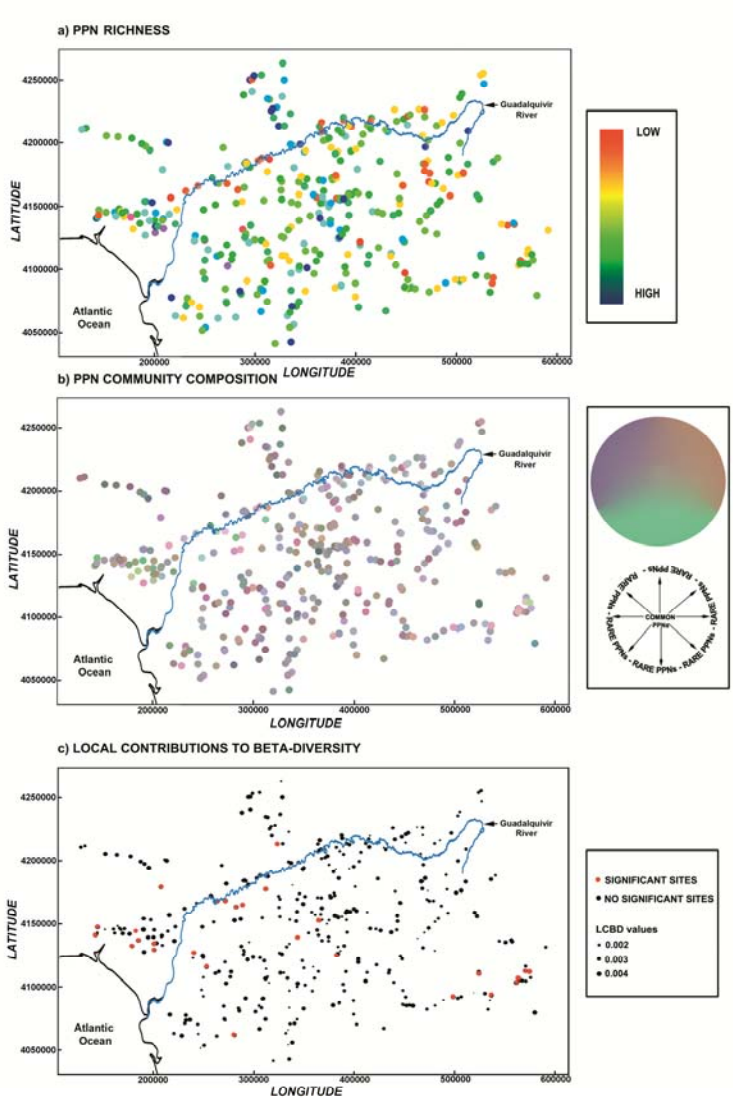


Figura 7.2: Maps of diversity indices used in the analysis. (a) Species richness, ranging from 2 (cyan) to 14 (dark blue). (b) Beta diversity map. Similar colours indicate similar species composition based on Bray-Curtis dissimilarity. (c) LCBD values map. LCBD values ranging from 0.0012 (small circles) to 0.0044 (large circles). No significant sites are indicated by black circles, and significant sites ($P < 0.005$) are indicated by red circles. In all map figures, the River Guadalquivir is indicated by blue line.

Table 7.1 Explanatory variables significantly related to variation of PPN community composition and species richness.

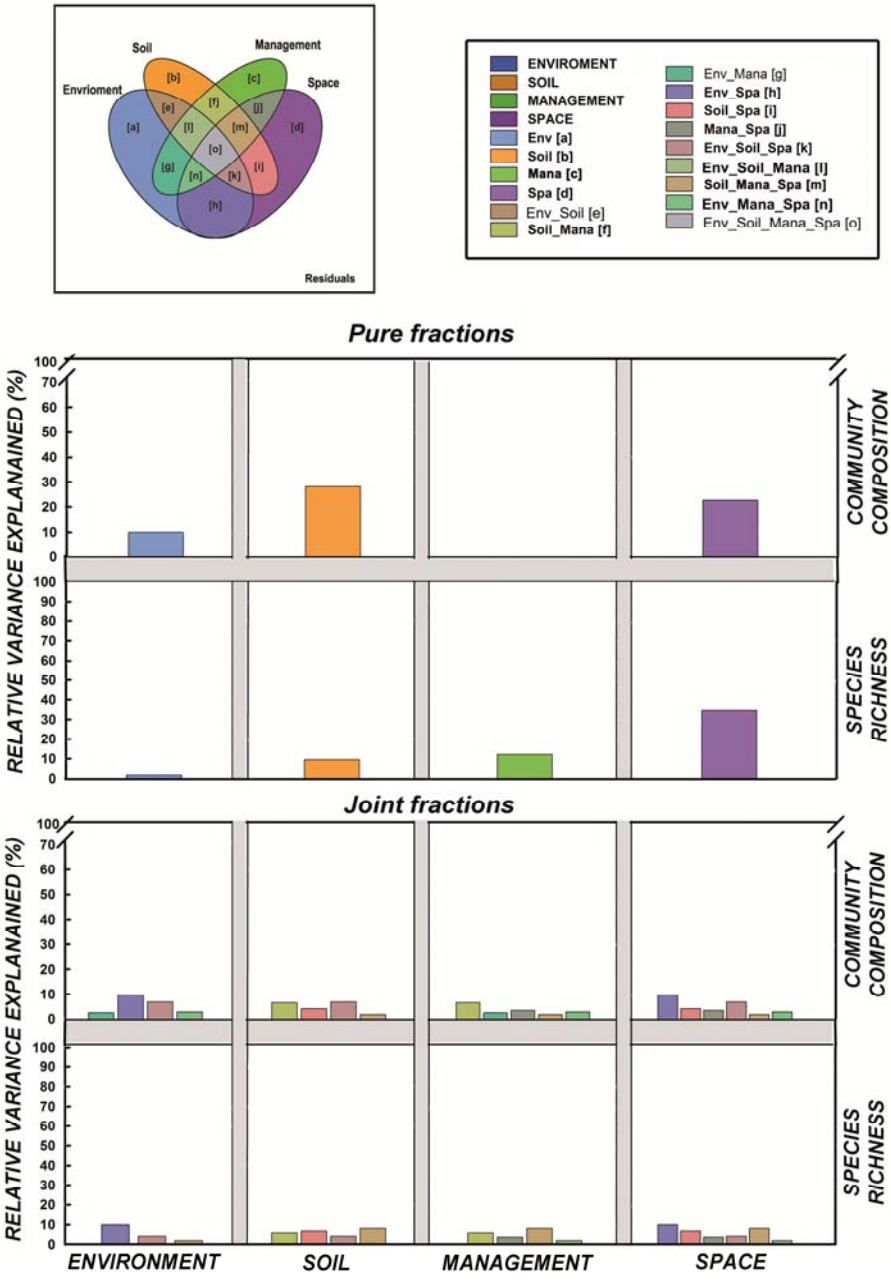
Variable	Community composition	Species richness
A) Environment		
BIO3	X	X
BIO4	X	-
BIO12	X	X
BIO18	X	-
Slope	X	-
Rainfall deficit	-	X
Climatic areas	5, 6	-
B) Soil		
CEC	X	-
Mg	X	-
Na	X	X
Pext	-	X
SOM	X	-
pH (KCl)	X	-
Soil Texture	LoSa	SaClLo
Soil edaphic unit	37, 44, 49, 52, 56	37, 47
C) Agronomic management		
Cultivar	"Arberquina", "Manzanilla Serrana", "Lechín"	-

Variable	Community composition	Species richness
Age	X	X
Irrigation	Irrigated	Irrigated
Canopy	-	Nothing
Alley	Vegetative cover	-
D) Spatial patterns		
PCNM variables	26, 2, 6, 1, 16, 19, 14, 31, 7, 23, 25, 44, 3	2, 14, 10, 6, 90, 51, 42, 9, 19, 44, 11, 52, 5

Notes: Significantly explanatory variables are indicated by X. For categorical variables (Supplemantary Information), selected predictors are indicated by category names of each significantly variables. Spatial patterns are described by PCNM variables (see Materials and Methods), indicating only the significantly PCNM variables for community composition and species richness.

3.3 Variation partitioning

Variation partitioning analysis revealed a high degree of stochasticity (or unaccounted environmental variables) in the variation of community composition and species richness. All four variable sets together explained about 15% of the variation of community composition and 32% of species richness patterns (Supplementary Information). Overall, we found a surprisingly large contribution of the joint fractions of the different sets of variables that represented more than half of the total contribution of each predictor set (i.e. pure + joint components). In addition, as shown in Figure 7.3, the relative (but not absolute) variance explained by the different sets of environmental variables and their joined effects were relatively similar between beta diversity and species richness (Figure 7.3).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Env_Soil [e]
Soil_Mana [f]
Env_Mana [g]
Env_Spa [h]
Soil_Spa [i]
Mana_Spa [j]
Env_Soil_Spa [k]
Env_Soil_Mana [l]
Soil_Mana_Spa [m]
Env_Mana_Spa [n]
Env_Soil_Mana_Spa [o]
Pure fractions
RELATIVE VARIANCE EXPLANAINED (%)
COMMUNITY COMPOSITION
SPECIES RICHNESS
Joint fractions
ENVIRONMENT
SOIL
MANAGEMENT
SPACE

Figura 7.3: Variation partitioning to explain the variation in community composition and species richness of plant-parasitic nematodes (PPN) based on variables describing the environment (Env), soil (Soil), agronomic management (Mana) and spatial patterns (Spa). The PPNs infest soils from cultivated olive in Andalusia (southern Spain). The proportion of relative variation explained by Env, Soil, Mana and Spa, split into pure and joint fractions (Env_Soil_Mana represent the joint fraction of environment, soil and agronomic anagement variables) is show as Venn diagram. The proportions of individual fractions sum to 100%. For clarity, the fractions with less than 1.5% of relative variance explained are not shown.

Most of the variation on both PPN community metrics (e.g. beta diversity and species richness) was spatially structured and the spatial variables explained in total 8.0 and 23.5% of the variation in species composition and species richness respectively, including pure and joint fractions. For species richness the pure space component [d] explained 11.2% (about 34% of the total variance explained), but showed a lower pure contribution on beta diversity (3%). The second most important component was soil that explained in total 13% of the variation in species richness and 7% of species composition. The pure (non-spatial) contribution of soil variables [b] explained 3.1% of the variation of species richness and 4% for species composition. The pure environment component [a] showed a low explanatory contribution because environmental variables tended to be spatially structured and related to patterns of the soil data set (Figure 3; Supplementary data). Finally, agronomic management variables explained 11% of the variation in species richness, but only 3% of species composition. The pure agronomic management component [c] had no effect on beta diversity, but explained about 4% of the variation in species richness.

3.4 Species and local contributions to beta diversity

The species contribution to beta diversity (SCBD) ranged from almost zero to 17%. Overall, migratory ectoparasitic PPN species including *Helicotylenchus digonicus* (SCBD = 0.1711), *H. vulgaris* (SCBD = 0.1164), *Xiphinema pachtaicum* (SCBD = 0.091476), and *H. oleae* (SCBD = 0.0792) showed the highest distribution heterogeneity found in our study. However, the majority of the remaining taxa were more homogeneously distributed

(SCBD < 0.002). SCBD was significantly related with nematode prevalence and density range (Table 7.2).

The local contribution to beta diversity (LCBD) exhibited a heterogeneity pattern across space (Figure 7.2c). Permutation test identified 27 orchards units with significant LCBD values. The highest and significant LCBD values were found mainly in olive orchards located on the western middle area and close to the downstream course of the Guadalquivir River (Figure 7.2c). However significant LCBD values were also located in the south-east area which is characterized by a semi-arid climate and markedly different from the rest of the study area (Rodrigo *et al.* 2012).

A simple linear regression model showed that LCBD was not significantly linearly related to PPN community metrics such as species richness and total nematode abundance in each olive orchard (Table 7.2). Therefore, large LCBD values may indicate sites that have rare species combinations or may be due to specific species ecological requirements. Partial linear regression and subsequently forward selection procedures (Legendre and Gauthier 2014) revealed that soil characteristics appeared to be more important at explaining LCBD (Table 7.3). The contribution of agronomic management characteristics was relatively moderate and further environmental variables had a marginal influence on LCBD.

Table 7.2 Results of regression analysis to explain the species contributions to beta diversity (SCBD) and the local contributions to beta diversity (LCBD) by PPN community indices.

	Estimate	SE	t value	P		R^2	R^2_{adj}
A) SCBD[1]							
(Intercept)	2.3 e-04	1.4 e-03	0.17	0.87			
Density range[2] (nem/ 500 cm^3)	2.5 e-06	5.6 e-07	4.5	1.6 e-05	***		
Prevalence (%) [3]	1.2e-03	1.2 e-04	10.3	< 2 e-16	***		
Model				< 2 e-16	***	0.58	0.57
B) LCBD[4]							
(Intercept)	2.7 e-03	1.9 e-04	14.1	< 2 e-06	***		
Total nematode abundance(biomass)	2.5 e-05	8.7 e-06	2.9	0.004	**		
Total nematode abundance(numeric)	-6.0 e-06	4.3 e-06	-1.4	0.16			
Species richness	-1.3 e-04	1.1 e-04	-1.2	0.23			
Model				0.01		0.03	0.02

[1] Result from the lineal regression model analysis based on Hellinger transformed abundance data form each PPN species.

[2] Density range index includes minimum and maximum density (nematodes/500 cm^3) detected by each PPN species.

[3] Prevalence was calculated by dividing the number of samples in which PPN species was detected by the total number of samples and expressed as a percentage.

[4] Results from the lineal regression model based on Hellinger transformed abundance data from each site.

Table 7.3 Forward selection procedure results of ecological predictors in explaining variation of LCBD (local contributions to beta diversity) values of plant-parasitic nematodes (PPN) infesting soils of olive orchards from cultivated olive in southern Spain.

		LCBD	
Ecological predictors 1, 2,3,4	**R^2**	**R^2_{adj} cum**	**P value**
Soil texture			
LoSa (loamy sand)	0.0552994	0.0527734	< 0.0001
pH (KCl)	0.0251797	0.0755487	0.0018
Soil edaphic unit			
I Re Lc Be	0.0133137	0.0864847	0.0129
Age of olive orchards	0.0150989	0.0993963	0.0119
Cultivar of olive orchards			
"Gordal"	0.0121414	0.1092668	0.0212
Soil edaphic unit			
Bv Vc Bk Rc	0.0119632	0.1190115	0.0225
Bk Bg Rc	0.0116259	0.1284646	0.0224
Cultivar of olive orchards			
"Lechín Granada"	0.0096819	0.1359829	0.0383
Climatic areas			
Sub-humid Atlantic Mediterranean	0.0098059	0.1436692	0.0367
Soil texture			
Sa (sand)	0.0096629	0.1512507	0.0382

[1] We used as ecological predictors the explanatory variables included in the environment, soil, and agronomic management data sets as whole.
[2] Order of explanatory variables is based on the R^2 values.
[3] See Supporting Information Appendix A for details of explanatory variables.
[4] Forward selection procedure was performed by controlling the effects of spatial component from the LCBD patterns using the PCNM variables as predictor set using partial linear regression with the indications described by (Legendre and Gauthier, 2014). (For more details, see Materials and Methods section).

4. Discussion

Understanding the drivers of community structure and species richness of plant-parasitic nematodes (PPN) is an essential task for both, increasing our general knowledge about ecological phenomena in soil ecosystems and for management of cropping systems (Bardgett and van der Putten 2014). Olive trees are of particular relevance because they host a large number of PPN and have a high economic and cultural importance (Castillo *et al.* 2010, Ali *et al.* 2017). In this study, we investigated the effects of different sets of environmental variables and pure spatial structure on the variation of community composition and species richness of PPN infesting the rhizosphere from cultivated olive in southern Spain. Our results revealed that spatial structure and soil were the most important factors driving communities of PPN, and that agronomic management practices contributed less than expected. However, stochasticity (or unaccounted environmental variables) accounted for 85% of the beta diversity and 67% of the variation in species richness.

4.1 The role of stochasticity on PPN community patterns

Variation partitioning has been used to assess the relative effect of deterministic and stochastic processes in plant communities (e.g., Svenning *et al.* 2004, Legendre *et al.* 2009, Amici *et al.* 2013, Baldeck *et al.* 2013a, Punchi-Manage *et al.* 2014), amphibians (Luiz *et al.* 2016), and below-ground communities (Dumbrell *et al.* 2009). For example, Baldeck *et al*. (2013a) found in a study on 25-50 ha plots of tropical forests (with resolution of 20 x 20m) that between 26 and 68% of the variation in species composition remained unexplained, and Amici *et al*. (2013) found for Mediterranean forests similar figures of between 28 and 58%. We found an even more prominent role of stochasticity in structuring PPN communities with 85% of the variation in species composition remaining unexplained (Supplementary Information). This finding reflects the large heterogeneity of the observed communities (Figure 7.2). Frequent disturbance of soils in agricultural systems may explain the relatively low contribution of deterministic processes (Ferrenberg *et al.* 2013) as opposed to natural

system where the variation of nematode community was more dominated by deterministic than stochastic processes (Moroenyane *et al.* 2016). However, the large proportion of unexplained variation may also be due to omission of exploratory variables and mechanism such as species interactions (De Cáceres *et al.* 2012). Additional factors influencing the relative importance of different mechanism on beta diversity may be the study scale as well as the spatial configuration and strength of ecological gradients used (Smith and Lundholm 2010).

4.2 Spatial structure of PPN community patterns

A recent meta-analysis by Soininen (2016) across major organism types and ecosystems showed that a mean of 11% of the variation in community composition was explained purely by spatial variables. We found here a rather low contribution of 3% for PPN, but a somewhat higher contribution for variation in species richness of 11% (Figure 7.3; Supplementary Information). The total contribution of spatial structure to variation in species richness and species composition was 24 and 8%, respectively. This suggests that PPN communities showed moderate levels of spatial structure caused by pure spatial structure and spatial gradients in explanatory variables.

The pure spatial component may represent the role of dispersal limitation of PPN within the soil ecosystem (De Cáceres *et al.* 2012). In a three-dimensional context, the movement capacity of soil nematodes is difficult to assess, but due to their small body size we can expect that they will actively move rather short distances (Gaugler and Bilgrami 2004). On the other hand, larger movement distances are possible, but not controlled by nematodes themselves but rather by agricultural activities (e.g. farm machinery, plant propagation material, or seeds) (Castillo *et al.* 2010, Neher 2010).

4.3 The role of the soil on PPN community patterns

Our study highlighted the influence of soil drivers in structuring PPN communities. Interestingly, the pure contribution of soil was somewhat larger for beta diversity (4.1%) than variation in species richness (3.1%), but there was a large shared contribution of soil with the other variables in species richness (9.4%) but not in beta diversity (3.5%). Thus, gradients in soil properties induce changes in PPN biomass and therefore, produce "habitat filtering" that reflect assemblage patterns of PPN largely independent of above-ground environmental gradients (in climate and topography).

Overall, soil chemical gradients showed among soil properties the strongest influence on spatial patterns of PPN communities (Table 7.1), including especially CEC, pH and Mg availability (Supplementary Information). The significant influence of cations ability to plant nutrition (that is, CEC) may likely to be the result of effect on PPN densities as a consequence of improved host-plant cations (Norton 1989), or direct effect on nematode populations based on a sensibility degree of nematodes to exchangeable cations (Norton 1978). In contrast to beta diversity, variation of species richness was strongly influenced by availability of P (Supplementary Information). Although P availability has been identified as a determinant on abundance of PPN (Norton 1978), other studies have revealed filtering effects on size of soil nematodes by P gradients (Mulder 2010). Specifically, variability of P concentration could structure PPN assemblages based on aggregation of nematodes to the entire community depending on size of PPN species (Gaugler and Bilgrami 2004).

As expected, soil physical parameters, including soil texture and soil type, were other major factors structuring beta diversity and variation of species richness (Table 7.1, Supplementary Information). Soil texture has been extensively studied on the distribution of soil nematodes identifying as important in driving patterns of PPN communities since the size of particles directly affects nematode movement (Norton 1989, Palomares-Rius *et al.* 2015). In this regard, our findings revealed a dissimilar texture class structuring both species richness and beta diversity (Table 7.1). This could suggest, assuming a suitable soil moisture film is necessary to nematode movement and activity; there is probably an optimal soil particle size for each nematode genera or species (Norton 1989, Palomares-Rius *et al.* 2015). In addition, the migration and activity of the PPN have been

described as being greater in soil with large than fine particle size which agrees with our findings about the significant effect of soils with loamy-sand texture class on beta diversity (Mateille *et al.* 2014, Palomares-Rius *et al.* 2015). On the other hand, we also found that young soils (e.g. regosols) and moderately developed soils (e.g. cambisols) as the main soil types structuring patterns of PPN communities (Table 7.1, Supplementary Information). One plausible explanation could be their high silt and sand content and the influence, therefore, on improvement of nematode activity (Godefroid *et al.* 2017).

4.4 The role of the environment on PPN community patterns

Overall, the variance explained by the unique contribution of environmental variables [a] (including climatic and topographic variables) was relatively small compared to that explained by the other sets of variables (Figure 7.3, Supplementary Information) and compared to analysis of plant communities. For example, Baldeck *et al*. (2013a) and Luiz *et al*. (2016) found in their studies on tropical forests and amphibian species that higher portion of variation of species composition was mainly explained by topographic and geomorphical variables, respectively. The relatively low contribution of environmental effects on PPN communities may be partly attributed to the scale at which this study was carried out. For instance, Nielsen *et al*. (2014) found that climate was strongly related with nematode assemblages at global scales, but no with local diversity descriptors. The negligible effect of topography may also be influenced by scale in relation to the range of elevation gradient (Dong *et al.* 2017).

Although the effect of the environmental set was negligible, we found that beta diversity and variation of species richness were specially related with annual mean precipitation and isothermality (BIO3; the ratio of the mean diurnal range to the annual temperature range). Based on the significant relationship between ishothermality and PPN communities, we may also confirm the effect of long-term warming on changes in nematode abundance (beta diversity) and in presence/absence of specific PPN species (species richness) (Bakonyi *et al.* 2007, Yan *et al.* 2017). Moreover, variation of species richness was related with extended period

time of rainfall deficit which may be associated with differing reactions of specific species in response to prolonged alterations in soil moisture (Bakonyi and Nagy 2000). Notwithstanding the influence of topography may be closely related with climatic drivers (De Cáceres *et al.* 2012), our results suggest that the effect of slope could be due to soil erosion processes which are more related with variation of nematode abundance than species richness (Tong *et al.* 2010).

4.5 The role of agronomic management practices on PPN community patterns

It is well documented that population dynamics of PPN in olive agroecosystems swiftly respond to agricultural management practices (Palomares-Rius *et al.* 2015, Sánchez-Moreno *et al.* 2015, Ali *et al.* 2017). However, our findings indicated no effect by the pure management component [c] on beta diversity (Figure 7.3, Supplementary Information). One plausible explanation could be the non-inclusion of spatial descriptors in previous studies so that the effect of management practices could be spatially structured (Vleminckx *et al.* 2017). In addition, the majority of these studies were not based on beta diversity approaches but on alpha or gamma diversity descriptors, which could show different trends.

The variation in species richness was associated with the pure management component (Figure 7.3, Supplementary Information) and related with the age of the olive plantation and the irrigation regimen in the orchard (Table 7.1, Supplementary Information). This result agrees with previous reports where changes in nematode community patterns were related to forest stand ages (Zhang *et al.* 2015). If conditions are suitable, we can expect that more nematode species will accumulate over longer periods of time (i.e., older olives) and additionally, larger canopies and optimum soil moisture (i.e., irrigation regimen) may improve the soil environmental conditions indirectly impacting PPN diversity (Caldeira *et al.* 2014). As expected, soil-vegetation cover was also important for structuring patterns of PPN communities both below the olive tree canopy (for species richness) and in alleys of olive orchards (for beta diversity) (Table 7.1, Supplementary Information). However, the influence of this agronomic practice on beta diversity was spatially structured and correlated with other soil or climatic factors since the no pure effect in this metrics (Figure 7.3).

The pure influence on species richness caused by natural herbaceous plants composing the soil cover below the tree canopy supports that plant species apply a relative selection of PPN at species level (Castillo *et al.* 2010, Neher 2010, Palomares-Rius *et al.* 2015). Other management practices, such as olive cultivar, weakly shaped beta diversity as their effect was spatially structured or correlated with other environmental factors (Figure 7.3, Supplementary Information). In particular, the filtering effect of olive cultivar on beta diversity is correlated with soil properties (fraction [f]) and/or depends of the spatial structure (fraction [j]) in contrast to the strong influence described by other authors (Palomares-Rius *et al.* 2015).

4.6 Contrast between species richness and species composition

Our study revealed substantial differences between the effects of overall environmental variables on beta diversity and species richness. First, the variation explained on species richness was higher than beta diversity, which is against the general pattern detected in above-ground systems where it was similar or the opposite trend (Legendre *et al.* 2009, Punchi-Manage *et al.* 2014). We suggest that gradients in habitat association modulating nematode abundance are exceptional heterogeneous in soil ecosystems which are strongly dominated by stochasticity. Thus, differences in the number of species detected in each sampling point may be more influenced by soil habitat factors, whereas neutral processes may strongly influence structure soil nematode abundance. Second, while the pure management component did not influence beta diversity, the pure component of management practices influenced variation of species richness. This is not surprising because some of PPN genera or specific species groups have shown sensitivity to agronomic practices (Castillo *et al.* 2010, Neher 2010, Palomares-Rius *et al.* 2015).

4.7 Ecological uniqueness of sites and species contributions to overall beta diversity of PPN

Species contributions to beta diversity (SCBD) was highly correlated with nematode occupancy (i.e. nematode prevalence and density range; R^2 =

57%) (Table 7.2). This suggests that SCBD could be related with niche position (Heino and Grönroos 2017). Therefore, dominant PPN species that show the largest abundance variation among sites may be a suitable indicator of fluctuations on specific environmental properties in agroecosystems (Heino and Grönroos 2017). We found that the PPN species with the highest SCBD index belonged to the genera *Helicotylenchus* and *Xiphinema* and were characterized by wide distributions and high prevalence as well as large abundance (Palomares-Rius *et al.* 2015, Archidona-Yuste *et al.* 2016a, Ali *et al.* 2017). In addition, the fact that the most of these species are migratory ectoparasitic and are characterized as "persister" nematodes make them suitable indicators to predict fluctuations of soil ecological properties or stable habitats (Bongers 1990).

We expected that the local contributions to beta diversity (LCBD) to be influenced primarily by species richness and nematode abundance (Heino and Grönroos 2017). However, these relationships were not observed in our study, but LCBD was positively related to total nematode biomass in each site, although this relationship was weak and curvilinear (adjusted R^2 = 0.02; Table 7.2). Biomass between PPN species can differ significantly (P<0.05) according to their body size (ranging from 200 to 12000 µm in body length) (Gaugler and Bilgrami 2004). Thus, LCBD may result in distinctly perceptible changes in PPN assemblages based on the close relationship between soil organism size and ecological gradients (Mulder 2010). Sites with significant values (highest LCBD index) showed nematode communities composed by rare PPN species (Figure 7.2b, c) as suggested by Legendre and De Cáceres (2013). Most of the sampling sites with large and significant LCBD values were grouped into two clearly separated areas (Figure 7c), suggesting strong habitat filtering mechanisms (Table 7.3).

5. Conclusions

Numerous studies have documented spatial structures in the distribution of plant and animal communities (Soininen 2016), but relatively little is known about how the diversity of soil organisms such as plant parasitic nematodes

(PPN) is structured. Here we found that PPN of cultivated olives showed moderate levels of spatial autocorrelation in the variation in species composition and species richness, as indicated by the fraction of variation of beta diversity and species richness explained by spatial descriptors. Soil variables were the second most influential factors driving communities of PPN, but agronomic management practices showed less influence than expected. Surprisingly, we found that more than two thirds of the variation remained unexplained, which is in contrast to common expectations that soil and management would mostly determine PPN community variation among sites. An interesting open question left for future studies is, if PPN of wild forms of olives would show more biological structure in beta diversity than found here for the cultivated forms. Our findings reveal novel insights for this group of soil fauna, and especially PPN, and show that their beta diversity is less structured by space and environmental factors as compared to other organism types such as plants or amphibian communities. In summary, novel insights are revealed about the pure effect of environment and agronomic practices on diversity and distribution of PPN infesting soils from agricultural ecosystems.

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8. Supplementary Information

Table 7.4 Forward selection procedure (based on permutation test with 9999 randomizations) results of each explanatory variables sets in explaining variation on community composition (beta diversity) of plant-parasitic nematodes (PPN) infesting soils of olive orchards from cultivated olive in Andalusia (southern Spain). The function forward.sel (*packfor* package in R freeware) was used for the forward selection (Dray 2011). The adjusted R^2 was used to reduce the inflated R^2 that is caused by the accumulation of explanatory variables (Borcard *et al.* 2011).

	Beta diversity			
Explanatory variables[1]	R^2	R^2 cum	R^2 adj cum	*P value*
Environment				
BIO12	0.0097	0.0097	0.0071	0.0001
BIO3	0.0090	0.0187	0.0135	0.0005
BIO4	0.0077	0.0265	0.0186	0.0022
CA				
Mediterranean moderate	0.0056	0.0321	0.0217	0.0125
Sub-humid Atlantic Mediterranean	0.0057	0.0378	0.0248	0.0154
BIO18	0.0051	0.0429	0.0274	0.025
Slope	0.0050	0.0479	0.0298	0.0317
Soil				
CEC	0.0146	0.0146	0.0119	0.0001
pH (KCl)	0.0104	0.0250	0.0198	0.0001
Na	0.0088	0.0338	0.0260	0.0004
Mg	0.0075	0.0413	0.0310	0.0031
Soil texture				
LoSa (Loamy Sand)	0.0066	0.0479	0.0351	0.0046
Soil edaphic unit				
Bk Rc I E	0.0055	0.0534	0.0380	0.0173
SOM	0.0055	0.0589	0.0410	0.0172
Soil edaphic unit				
Bv Vc Bk Rc	0.0049	0.0638	0.0434	0.0289
Lc Bk I	0.0049	0.0688	0.0459	0.0239
Lc Be I	0.0046	0.0734	0.0480	0.0316
Be Lc Lo	0.0044	0.0779	0.0500	0.0427
Agronomic management				
Irrigation				
Irrigated	0.0110	0.0110	0.0084	0.0002
Age	0.0086	0.0196	0.0144	0.0012
Cultivar				
“Manzanilla Serrana”	0.0060	0.0257	0.0178	0.0099
Alley				
Vegetative cover	0.0049	0.0305	0.0201	0.0371
Cultivar				
“Lechín”	0.0047	0.0352	0.0222	0.0462
“Arberquina”	0.0048	0.0400	0.0244	0.0387

	Beta diversity			
Explanatory variables[1]	R^2	R^2_{cum}	$R^2_{adj\ cum}$	***P value***
V26	0.0102	0.0102	0.0075	0.0001
V2	0.0095	0.0196	0.0144	0.0001
V6	0.0078	0.0275	0.0196	0.0013
V1	0.0071	0.0346	0.0242	0.0028
V16	0.0066	0.0412	0.0282	0.0051
V19	0.0062	0.0474	0.0319	0.0077
V14	0.0062	0.0536	0.0356	0.0082
V31	0.0059	0.0594	0.0389	0.0105
V7	0.0052	0.0647	0.0417	0.0198
V23	0.0049	0.0695	0.0440	0.0315
V25	0.0048	0.0743	0.0463	0.0291
V44	0.0047	0.0790	0.0486	0.0368
V3	0.0045	0.0835	0.0506	0.0414

[1] Order of explanatory variables is based on the R^2 values.

[2] Significant variables from the all the positive eigenfunctions derived from PCNM analysis.

Table 7.5 Forward selection procedure (based on permutation test with 9999 randomizations) results of each explanatory variables sets in explaining variation of species richness of plant-parasitic nematodes (PPN) infesting soils of olive orchards from cultivated olive in Andalusia (southern Spain). The function forward.sel (*packfor* package in R freeware) was used for the forward selection (Dray 2011). The adjusted R^2 was used to reduce the inflated R^2 that is caused by the accumulation of explanatory variables (Borcard *et al.* 2011).

	Species richness			
Explanatory variables[1]	R^2	R^2 cum	R^2 adj cum	*P value*
Environment				
BIO12	0.0426	0.0426	0.0401	0.0001
BIO3	0.0187	0.0613	0.0563	0.0067
RD (Rainfall deficit)	0.0110	0.0724	0.0649	0.0398
Soil				
P_{ext}	0.0686	0.0686	0.0661	0.0001
Na	0.0230	0.0916	0.0868	0.0027
Soil texture				
SaClLo (Sandy clay loam)	0.0214	0.1131	0.1059	0.0032
Soil edaphic unit				
Be Lc Lo	0.0137	0.1268	0.1173	0.0161
Bk Lk Lc Jc	0.0100	0.1367	0.1251	0.0421
Agronomic management				
Irrigation				
Irrigated	0.0618	0.0618	0.0593	0.0001
Canopy				
Nothing	0.0275	0.0894	0.0845	0.0007
Age	0.0233	0.1126	0.1055	0.0014
Spatial patterns[2]				
V2	0.0463	0.0463	0.0437	0.0001
V14	0.0298	0.0760	0.0711	0.0005
V10	0.0286	0.1046	0.0974	0.0003
V6	0.0263	0.1309	0.1216	0.0009
V90	0.0225	0.1535	0.1420	0.0021
V51	0.0202	0.1737	0.1602	0.0031
V42	0.0151	0.1888	0.1733	0.0093
V9	0.0132	0.2020	0.1846	0.0133
V19	0.0130	0.2150	0.1957	0.0131
V44	0.0124	0.2274	0.2062	0.0164
V11	0.0123	0.2396	0.2167	0.0166
V52	0.0119	0.2516	0.2268	0.01681
V5	0.0103	0.2619	0.2354	0.02621

[1] Order of explanatory variables is based on the R^2 values.

[3] Significant variables from the all the positive eigenfunctions derived from PCNM analysis.

Table 7.6 Results of redundancy analyses (RDA) and subsequent variation partitioning for community composition and species richness of plant- parasitic nematodes (PPN) infesting soils of olive orchards from cultivated olive in Andalusia (southern Spain). Each row indicates the variance explained by pure and joint fractions as well as among them together of environment (Env), soil (Soil), agronomic management (Mana), and spatial patterns (Spa) variables, total variance explained by all fractions (All) and unexplained variance (Residuals). Symbols of fractions, in squared brackets, are visualized in the Venn diagram in Figure 7.3. Analyses were performed separately for community composition and species richness. Percentages of variance explained by pure and joint fractions are given as R^2 adjusted (R^2_{adj} *100).

		Variance explained for the PPNs communities	
Fractions	**Symbols[1]**	**Community composition (%)**	**Species richness (%)**
Pure + joint fractions			
Environment		0.05028	0.06488
Soil		0.07323	0.12507
Management		0.03077	0.10549
Spatial pattern		0.08017	0.23538
Pure fractions			
Env	[a]	0.01446	0.00552
Soil	[b]	0.04107	0.03063
Mana	[c]	-	0.03870
Spa	[d]	0.03311	0.11227
Joint fractions			
Env_Soil	[e]	-	0.00352
Soil_Mana	[f]	0.00950	0.01870
Env_Mana	[g]	0.00349	-
Env_Spa	[h]	0.01408	0.03198
Soil_Spa	[i]	0.00602	0.02181
Mana_Spa	[j]	0.00494	0.01153
Env_Soil_Spa	[k]	0.00992	0.01320
Env_Soil_Mana	[l]	0.00162	-
Soil_Mana_Spa	[m]	0.00244	0.02590
Env_Mana_Spa	[n]	0.00406	0.00629
Env_Soil_Mana_Spa	[o]	0.00559	0.01240
All		**0.14649**	**0.32442**
Residuals		**0.85351**	**0.67558**

[1]Symbols for each explanatory variable data set including pure and joint fractions: Environment [aeghklno], Soil [befiklmo], Management [cfgjlmno], and Spatial pattern [dhijkmno].

8.1 References Supplementary Information

Borcard, D., Gillet, F. & Legendre, P. (2011) *Numerical Ecology with R*. Springer New York.

Dray, S. (2011) packfor: forward selection with permutation (Canoco p.46).

8 DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

Descifrar la biodiversidad de los nematodos que habitan el suelo es una tarea esencial para el incrementar el conocimiento sobre procesos ecológicos que subyacen del desarrollo de la evolución, aspectos biogeográficos y físicos en los ecosistemas tanto bajo como sobre el suelo (Bardgett y van der Putten 2014). En un contexto agrícola donde la presencia de aquellas especies capaces de parasitar las plantas pueda llegar a ser un problema fitopatológico, llevar a cabo estudio sistemático de esta naturaleza es una de las claves de éxito para el diseño y aplicación de adecuadas prácticas de manejo (Freckman y Caswell 1985, van der Putten *et al.* 2006). Son diversos los motivos que enfatizan la eficacia del enfoque sistemático, aunque todos están relacionados con la capacidad de señalar los factores ambientales que determinan la variabilidad de los patrones espaciales de los nematodos fitoparásitos (NF) con un mayor grado de credibilidad (Ingram 1999, Swift *et al.* 2004). Todo ello está positivamente influenciado por los avances substanciales ocurridos en los últimos años en el desarrollo de medidas que permiten cuantificar la heterogeneidad espacial de la biodiversidad (Gaston 1994, Purvis y Hector 2000, Gaston 2009, Anderson *et al.* 2011, Baselga y Chao 2017, Wiegand *et al.* 2017), sin olvidar los destacados avances en la biología molecular en el desarrollo de técnicas de análisis genético de comunidades biológicas (Purvis y Hector 2000). Además, se debe mencionar, de manera especial, que los avances en la diversidad beta son clave dado el excepcional atributo e idoneidad para evaluar cuantitativa- y cualitativamente los procesos locales y regionales ($\beta = \gamma/\alpha$, (Whittaker 1960); Recuadro 1) que estructuran la variabilidad espacial de las comunidades de especies (Tuomisto 2010, Vellend 2010, Anderson *et al.* 2011). En este sentido, la notable variabilidad agronómica y ecológica, basada en la amplitud de los gradientes ambientales, que caracterizan el agroecosistema del olivar andaluz lo convierten en un escenario ideal para realizar un estudio sistemático de la biodiversidad de NF así como testar hipótesis sobre los factores que determinan sus patrones con el fin de aplicar un manejo eficiente, y de esta manera incrementar el conocimiento sobre las reglas ecológicas que determinan la distribución espacial de estos organismos de suelo en los agroecosistemas.

Desde que se detectó la primera especie de un NF asociada a la rizosfera de olivo (Buhrer *et al.* 1933), la biodiversidad de NF asociada al cultivo del olivo ha aumentado considerablemente en los últimos años. En la bibliografía son diversos los estudios que han resaltado y aceptado este incremento (Lamberti y Vovlas 1993, Sasanelli 2009, Castillo *et al.* 2010, Ali *et al.* 2014). Las posibles causas de este hecho pueden estar relacionadas con la elevada capacidad del olivo para hospedar una amplia variabilidad de NF y el atributo polífago de la mayoría de las especies encontradas en asociación con el olivo (Perry y Moens 2006, Castillo *et al.* 2010, Sikora *et al.* 2018), sin olivar los extraordinarios avances en la identificación taxonómica mediante las técnicas moleculares (Palomares-Rius *et al.* 2017b, Seesao *et al.* 2017). Además, se debe mencionar que existen ciertos casos donde la relación biológica entre huésped y parásito se encuentra distribuida ampliamente por las zonas olivareras más importantes. Un ejemplo de ello lo encontramos para la especie *Helicotylenchus oleae* y su amplia distribución detectada infestando la rizosfera de olivo en las zonas olivareras más relevantes de la Cuenca Mediterránea (Palomares-Rius *et al.* 2018b), además de otras especies con una alta importancia económica como *Meloidogyne javanica* entre otras (Castillo *et al.* 2010, Ali *et al.* 2014, Archidona-Yuste *et al.* 2018, Palomares-Rius *et al.* 2018a).

Como ya se ha descrito anteriormente en la introducción de esta tesis, la revisión bibliográfica realizada por Ali *et al.* (2014) mostró y confirmó la excepcional aptitud del olivo por hospedar una extensa y amplia variabilidad de especies de NF. Posteriormente el estudio basado en prospecciones sistemáticas realizado en la zona olivarera de Marruecos incrementó el número de especies asociadas al olivo desde 153 hasta 223 especies de NF a nivel mundial (Ali *et al.* 2017). Este notable incremento de la diversidad asociada al olivo se debe en mayor parte al enfoque científico basado en prospecciones sistematizadas con el fin de obtener resultados factibles al respecto, lo cual ensalza su importancia en los estudios de biodiversidad. En este contexto, los resultados obtenidos al respecto provienen de la aplicación de un enfoque sistemático y caracterizado además, por una identificación taxonómica integrativa (a nivel de especie) mediante una metodología que integra la identificación morfológica mediante herramientas microscópicas así como el uso de

herramientas moleculares (Castillo *et al.* 2003, Cantalapiedra-Navarrete *et al.* 2013, Gutiérrez-Gutiérrez *et al.* 2013b, Palomares-Rius *et al.* 2017b). De hecho, se han realizado prospecciones nematológicas en un total de 376 parcelas de olivar cultivado ampliamente distribuidas por toda la superficie de este cultivo en Andalucía, lo que supone el estudio científico con el mayor esfuerzo en cuanto al muestreo realizado hasta la fecha. Es por ello por lo que los resultados son sorprendentes, superando, a priori, las expectativas establecidas por lo descrito en los estudios anteriores realizados en este sentido (Castillo *et al.* 2010, Ali *et al.* 2014, 2017, Palomares-Rius *et al.* 2015). En total se han identificado hasta 128 especies de NF pertenecientes a 38 géneros asociadas al olivo, superando la diversidad detectada en un estudio previo realizado en la misma zona (70 especies, Palomares-Rius *et al.* 2015), y la observada en el estudio sistemático llevado a cabo en Marruecos (117 especies; Ali *et al*. 2017). Este hecho corrobora la hipótesis citada anteriormente que señalaba la falta de información en cuanto a la diversidad de especies encontrada hasta la fecha, ya que en el estudio previo no se consideró en su totalidad la variabilidad de sistemas de cultivo así como la extensa distribución de este cultivo en Andalucía (Palomares-Rius *et al.* 2012, 2015). No obstante, es necesario destacar que existen diversas especies detectadas en ciertos puntos de muestro que no han sido consideradas en los análisis de la riqueza de especies total del estudio, dada su dudosa capacidad de poder parasitar plantas, y en concreto el olivo. Este es el caso de las especies del género *Filenchus*, caracterizadas por ser especies que se alimentan de hongos en su gran mayoría, aunque se ha citado que ciertas especies tienen capacidad de parasitar plantas, aunque este aspecto que no está aclarado hasta la fecha y necesita ser estudiado (Okada *et al.* 2005). Por otro lado, otras especies de NF tales como *Heterodera avenae, Pratylenchus neglectus, Pratylenchus thornei*, *Zygotylenchus guevarai* u otras especies de los géneros *Ditylenchus*, *Heterodera* y *Globodera* se han tenido en cuenta a pesar que el olivo no es un huésped adecuado para ellas. Sin embargo, se detectaron en la rizosfera de olivo y podrían estar asociadas a otras especies de plantas que crecen de forma natural en los campos de olivo o como aquellas incluidas en las cubiertas vegetales (Castillo *et al.* 2010).

La nematofauna y distribución de NF que infestan suelos de campos de olivo ha sido analizada previamente en estudios efectuados en importantes zonas olivareras de la Cuenca Mediterránea (Palomares-Rius *et al.* 2015, Ali *et al.* 2017). Como se ha expuesto en el párrafo anterior, estos estudios detectaron una notable diversidad de NF asociados con el olivo, lo que concuerda con la notable capacidad del olivo como huésped de una gran variedad de NF (Castillo *et al.* 2010, Ali *et al.* 2014). Una explicación para ello fue propuesta por Ali y colaboradores (2017) que formularon la hipótesis de la existencia de una relación entre el número de especies detectadas con el esfuerzo de muestreo como una de las principales razones en la alta diversidad encontrada. En este sentido no se puede considerar este razonamiento como novedoso ya que describe una de las leyes fundamentales establecidas en el campo de la ecología, la relación especie-área (SAR) y el esfuerzo de muestreo (SE); la riqueza de especies depende tanto del área de estudio considerada como del número y distribución de los puntos de muestreo (Azovsky 2011). De hecho, las prospecciones sistemáticas llevadas a cabo en este estudio, si recordamos basada en el mayor esfuerzo de muestreo en olivos hasta la fecha, ha dado como resultado la mayor diversidad taxonómica de NF registrada en este cultivo (Ali *et al.* 2014, Palomares-Rius *et al.* 2015, Ali *et al.* 2017). En consecuencia, el presente estudio incrementa además de manera considerable la variabilidad de nematodos asociados con la planta de olivo, estimándose en un total de 250 especies documentadas a nivel mundial (Lamberti y Vovlas 1993, Sasanelli 2009, Castillo *et al.* 2010, Ali *et al.* 2014, 2017, Palomares-Rius *et al.* 2015).

Un total de 13 familias de nematodos han sido identificadas en las 128 especies identificadas en este estudio, el número de especies por familia varió entre una especie detectada como en el caso de la familia Rotylenchulidae a 28 especies para la familia Longidoridae. En general, los patrones observados en este estudio respecto a la distribución de las especies detectadas por género fueron muy similares a aquellos reportados igualmente en la zona olivarera de Andalucía (Palomares-Rius *et al.* 2015) o en Marruecos (Ali *et al.* 2017). Sin embargo, los resultados obtenidos en este estudio muestran una mayor diversidad de especies pertenecientes a la familia Longidoridae, la cual puede considerarse como un aspecto destacable en cuanto al número de especies y a la variabilidad

morfológica detectada. Estos datos confirman la hipótesis formulada con anterioridad, la cual apuntaba a una diversidad de especies de nematodos longidóridos similar a la observada para el cultivo de la vid en un estudio realizado también en la Cuenca Mediterránea, dadas las similitudes entre el olivo y la vid en contexto agronómico y de superficie ocupada en la zona (Rallo 1998, Perry y Moens 2006, Gutiérrez-Gutiérrez 2011, Navas-Cortés y Castillo 2014, Sikora *et al.* 2018). No obstante, los aspectos más relevantes sobre los nematodos longidóridos en cuanto a diversidad, distribución y prevalencia detectados en el olivo en Andalucía (**Bloque II, "La familia Longidoridae en Olivo en Andalucía" de la presente Tesis Doctoral**) serán discutidos más adelante, dada su novedad en cuanto a la escasez de estudios en este sentido. Por otro lado, se debe mencionar que otros grupos de nematodos caracterizados por su impacto económico como son aquellos que pertenecen al género *Meloidogyne* también mostraron una destacable diversidad taxonómica. Hasta seis especies de nematodos noduladores fueron detectadas en la rizosfera de olivo, de las cuáles cinco son ampliamente conocidas (*M. arenaria, M. baetica, M. hapla, M. incognita, M. javanica*) y una recientemente descrita como *M. oleae* (Archidona-Yuste *et al.* 2018). Aunque varios estudios han sido enfocados en la incidencia de nematodos noduladores en los plantones de olivo en los viveros de Andalucía (Nico 2002), la información acerca de la incidencia de estas especies en el olivar andaluz ha sido insuficiente y escasa a lo largo de los años. Sin embargo, un estudio reciente ha proporcionado por primera vez información detallada sobre diversidad, prevalencia, distribución y niveles de población de especies de *Meloidogyne* infectando el olivo en Andalucía, incluyendo tanto olivos silvestres como cultivados (Archidona-Yuste *et al.* 2018). Los datos obtenidos son sorprendentes en cuanto a la prevalencia detectada, ya que sólo alrededor del 7% de los puntos de muestro (499) dieron positivo en la presencia de alguna especie de este género (Archidona-Yuste *et al.* 2018).

Otros datos interesantes obtenidos en este estudio han sido la densidad de población y prevalencia de cada una de las 128 especies de NF identificadas (**Bloque I, "Nematodos fitoparásitos asociados al cultivo del olivo en Andalucía"**). En este sentido los resultados obtenidos también fueron similares a aquellos descritos en estudios previamente realizados en la Cuenca Mediterránea (Palomares-Rius *et al.* 2015, Ali *et*

al. 2017). Como podemos observar en el **Capítulo 1**, las tres familias más prevalentes fueron Tylenchidae, Paratylenchidae y Criconematidae. Los nematodos ectoparásitos migratorios tales como *Helicotylenchus digonicus*, *Merlinius brevidens* y *Xiphinema pachtaicum* mostraron los índices más altos de prevalencia, llegando incluso a estar presente en más del 70% de las parcelas de olivo muestreadas. La notable prevalencia observada en estas especies era la esperada ya que concuerda con los resultados reportados previamente (Palomares-Rius *et al.* 2015, Ali *et al.* 2017). Además, la incidencia de estas especies está ampliamente distribuida en las zonas olivareras de la Cuenca Mediterránea (Castillo *et al.* 2010, Ali *et al.* 2014, 2017, Palomares-Rius *et al.* 2015, Tzortzakakis *et al.* 2018). Los resultados obtenidos aquí podrían apoyar la hipótesis de que la especie *X. pachtaicum* podría ser un problema de impacto económico real en las zonas olivareras (Peña-Santiago 1990) ya que es posible que las densidades de población presentes en campo puedan ser superiores a aquellas detectadas en los estudios realizados en condiciones controladas (Hashim 1983). En este sentido, la abundancia total de nematodos detectados en cada parcela de olivo muestreada varió entre tan sólo 7 especímenes hasta llegar casi a los 20,000 ejemplares por 500 cm^3 de suelo, siendo las familias Meloidogynidae, Hoplolaimidae y Paratylenchidae las que presentaron los datos más elevados sobre promedio de densidad de población. Al igual que en los datos de prevalencia, especies de nematodos ectoparásitos, *Helicotylenchus oleae* y *Ogma rhombosquamatum*, mostraron las densidades de población más elevadas llegando hasta los 19,000 especímenes por 500 cm^3 de suelo para el primer caso (para más detalle ver **Capítulo 1** ("**Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in southern Spain").** No obstante, no sólo especies ectoparásitas fueron encontradas en densidades altas de población ya que nematodos endoparásitos como *Meloidogyne javanica* fue encontrada en una densidad de 10,000 nematodos por 500 cm^3 de suelo, lo cual podría producir una importante pérdida de producción en el olivo (Castillo *et al.* 2010).

El segundo objetivo específico de la presente Tesis Doctoral está relacionado con el estudio de la diversidad y distribución de especies de nematodos pertenecientes a la familia Longidoridae mediante un enfoque

multidisciplinar que caracteriza esta investigación, relacionando el estudio de la diversidad y distribución de estas especies en olivo en Andalucía. Como ya se ha explicado anteriormente, la capacidad como vectores de virus por parte de algunas especies de nematodos longidóridos (Nicol *et al.* 2011), la capacidad potencial de producir una merma en la producción hasta del 10% (Ali *et al.* 2014), unido a la escasa información que existe sobre la diversidad de estos nematodos infestando suelos de olivo en Andalucía, suscita el interés de su estudio. Además, la diversidad detectada en este trabajo con la identificación de un total de 28 especies diferentes (9 especies pertenecientes al género *Longidorus* y 19 a *Xiphinema*), siendo la familia de nematodos con una mayor representación en la nematofauna total detectada, justifica aún más si cabe el establecimiento de dicho objetivo de la presente Tesis Doctoral. Si realizamos una revisión bibliográfica al respecto, sólo los datos de diversidad detectados en el cultivo de la vid a su vez en Andalucía con la identificación de 32 especies (Gutiérrez-Gutiérrez 2011) puede superar la diversidad de nematodos longidóridos encontrada en este estudio. Además, no existen estudios que hayan detectado tal diversidad de este grupo de nematodos asociados al cultivo del olivo, considerándose está como excepcional y una novedad (Castillo *et al.* 2010, Ali *et al.* 2014, 2017, Palomares-Rius *et al.* 2015). No obstante, como se ha indicado en la parte donde se detalla la estructura y objetivos de esta Tesis Doctoral (**epígrafe 1.5 de Introducción General y Objetivos**), en este trabajo también se ha estudiado la diversidad de nematodos longidóridos asociados al olivar silvestre presente en Andalucía. Por lo tanto, si consideramos la planta del olivo en sus dos formas, cultivada y silvestre, la diversidad de este grupo de nematodos es incrementada sustancialmente llegando hasta un total de 45 especies diferentes (13 especies para el género *Longidorus* y 32 para *Xiphinema*). Dada la extraordinaria diversidad de especies de la familia Longidoridae detectada en este estudio, el sur de la Península Ibérica (concretamente en Andalucía) podría considerarse como un área de especiación de nematodos longidóridos al igual que se ha descrito para otro grupo de nematodos también pertenecientes al orden Dorylaimida, la familia Trichodoridae (Decraemer *et al.* 2013). No obstante, este aspecto debería ser profundizado mediante estudios específicos de biogeografía teniendo en cuenta varias áreas, y a escala global, donde el esfuerzo de

muestreo sea similar y dentro un contexto sistemático (Meza *et al.* 2011, Cameron *et al.* 2018).

La familia Longidoridae se caracteriza por presentar una extraordinaria diversidad morfológica entre las especies del mismo y diferentes géneros (Decraemer y Robbins 2007). A esto hay que añadir el elevado número de especies descritas hasta la fecha, llegando alcanzar hasta más de 260 especies en el género *Xiphinema* (Gutiérrez-Gutiérrez *et al.* 2013b, Tzortzakakis *et al.* 2015, Peraza-Padilla *et al.* 2018). Es tal la diversidad morfológica que podemos encontrar en el género *Xiphinema* que fue dividido en dos grupos de especies con el fin de facilitar el diagnóstico taxonómico (Loof y Luc 1990, Lamberti *et al.* 2000, Coomans *et al.* 2001): (1) el grupo *Xiphinema americanum*, que comprende un complejo de aproximadamente 55 especies caracterizadas por diversos caracteres morfológicos destacando la longitud del cuerpo medio en forma de C y sistema reproductivo femenino con dos ramas genitales igualmente desarrolladas sin diferenciación uterina y una cola cónica corta normalmente convexa-conoide y corta; y (2) el grupo *Xiphinema* non-*americanum*, que comprende un complejo de más de 200 especies caracterizadas normalmente por una longitud de estilete y cuerpo mayor, y sistema reproductivo femenino ampliamente variable entre especies (p.ej. ramas genitales desigualmente desarrolladas, diferenciación uterina, estructuras en la parte tubular del útero, etc.). A su vez, la extensa diversidad morfológica que exhibe el grupo *X.* no-*americanum* es dividida en un total de 8 grupos de especies diferenciados entre sí por la variabilidad de en el sistema reproductivo femenino, es decir, grupos de morfo-especies (Loof y Luc 1990, Coomans *et al.* 2001, Peraza-Padilla *et al.* 2018).

Todo lo expuesto con anterioridad refleja la complejidad que puede existir a la hora de proceder con la identificación de una especie de nematodos ectoparásitos migratorios de la familia Longidoridae. A esto hay que incluir que es probable que el número de especies siga aumentando conforme avanzan los años y las técnicas de diagnóstico siguen progresando. Además es necesario recordar que los nematodos son considerados los organismos de suelo con el menor porcentaje de especies descritas respecto al estimado (Decaëns *et al.* 2006, Wall *et al.*

2012, 2015, Phillips *et al.* 2017). En definitiva, el proceso de identificación taxonómica en este grupo de nematodos es realmente complejo y complicado, el cuál puede consumir un largo periodo de tiempo para realizar una identificación concisa y fiable. Por otro lado, la aplicación de métodos moleculares con el fin de determinar la estructura sistemática de poblaciones de nematodos longidóridos ha revelado un alto grado de presencia de complejos de especies crípticas, morfológicamente indistinguibles pero filogenéticamente distintas entre sí (Ye y Robbins 2005, Oliveira *et al.* 2006, Wu *et al.* 2007, Barsi y Luca 2008, Gutiérrez-Gutiérrez *et al.* 2010, 2013a, 2013b, Peraza-Padilla *et al.* 2016). Aunque ya ha sido mencionado en diversas ocasiones, las secuencias de ADN ribosómico nuclear (ADNr) y ADN mitocondrial (ADNmt) han sido intensamente utilizadas durante la última década para la caracterización molecular y reconstrucción de relaciones filogenéticas dentro de la familia Longidoridae, y en particular en el género *Xiphinema* (De Luca *et al.* 2004, He *et al.* 2005, Ye y Robbins 2005, Gutiérrez-Gutiérrez *et al.* 2010, 2012, 2013a, 2013b, Peneva *et al.* 2013, Subbotin *et al.* 2014, Tzortzakakis *et al.* 2014, Tzortzakakis *et al.* 2015, Guesmi-Mzoughi *et al.* 2017, Peraza-Padilla *et al.* 2018). En este sentido, se han sido utilizados varios genes para caracterizar molecularmente especies de nematodos longidóridos, proporcionando un elemento adicional a las herramientas clásicas de caracterización morfológica incrementando así la fiabilidad y eficiencia en el proceso de diagnóstico. Brevemente, las secuencias más utilizadas han sido la región D2D3 del gen ribosómico 28S, la secuencia ribosómica correspondiente al espacio interno transcrito (ITS), el gen 18S, y los genes mitocondriales subunidad 1 de la citocromo c oxidasa (coxI) y nicotinamida deshidrogenasa 4 (nad4) (Ye *et al.* 2004, He *et al.* 2005, Gutiérrez-Gutiérrez *et al.* 2013b, Subbotin *et al.* 2014, Zasada *et al.* 2014, Peraza-Padilla *et al.* 2016, 2018, Palomares-Rius *et al.* 2017b). También cabe señalar que el uso de estas secuencias en la aplicación del método taxonómico “barcoding” ha mostrado óptimos resultados para el diagnóstico de especies en la familia Longidoridae (Palomares-Rius *et al.* 2017b). No obstante, los mejores resultados han sido obtenidos para la región ITS debido a la clara variabilidad molecular existente entre especies para este marcador molecular (Palomares-Rius *et al.* 2017b).

Con la ayuda de las técnicas moleculares unido a la aplicación de métodos clásicos de morfología, en el presente estudio se han diagnosticado un total de 48 especies diferentes de la familia Longidoridae (ver **Bloque II, La familia Longidoridae en el olivo de Andalucía**). Cabe señalar que dada la participación en diversos proyectos de investigación durante el periodo de ejecución de la presente Tesis Doctoral, tres del total de las especies de nematodos longidóridos diagnosticadas no fueron detectadas parasitando la rizosfera de la planta de olivo (ya sea en su forma silvestre o cultivada). En definitiva, se ha demostrado el uso combinado de las distintas regiones de ADNr como la mejor estrategia, junto con la morfología, a seguir en estudios taxonómicos de especies de nematodos longidóridos. Esta aplicación polifásica en el proceso de diagnóstico de nematodos proporciona nuevos conocimientos que ayudarán a clarificar la distribución de este complejo de especies, que además ayudará a facilitar el diagnóstico en futuros trabajos de investigación. De hecho, se ha confirmado el uso de las secuencias ribosómicas relacionadas con las regiones D2D3 e ITS como una potente herramienta en la identificación de especies (Gutiérrez-Gutiérrez 2011, Archidona-Yuste *et al*. 2017a,b). Sin embargo, al igual que en estudios previos se han obtenido mejores resultados en cuanto a relaciones filogenéticas cuando se ha utilizado la región D2D3. En este sentido, la gran diversidad molecular detectada entre especies tanto en el género *Longidorus* como *Xiphinema* para la región ITS1 sugiere que una gran variedad de factores desconocidos a día de hoy están involucrados en la rápida evolución de esta región en los nematodos. Las relaciones filogenéticas basadas en la región D2D3 a partir de las especies de nematodos longidóridos identificadas en este estudio junto con las ya depositadas en las bases de datos moleculares (p.ej."GenBank") de las especies de longidóridos han revelado nuevos conocimientos sobre los procesos evolutivos en la familia Longidoridae, especialmente para el género *Longidorus*. Por ejemplo, las relaciones filogenéticas observadas en el género *Longidorus* revelaron agrupaciones de especies soportadas adecuadamente revelando cierta cercanía evolutiva entre las especies, destacando aquel grupo de especies mayormente endémicas de la Península Ibérica (**Capítulo 5 "Unravelling the biodiversity and molecular phylogeny of needle neamatodes of the genus *Longidorus* (Nematoda: Longidoridae) in Olive and a description of six new**

species"), lo cual corrobora la hipótesis que establece el papel relevante de ésta zona en la especiación y diversidad de especies de éste género (Gutiérrez-Gutiérrez 2011).

Las relaciones filogenéticas observadas en el caso del género *Xiphinema* no revelaron relaciones significativas entre los clados delimitados con características geográficas y/o propiedades intrínsecas de las especies que lo componen. Particularmente, es relevante resaltar que este aspecto ha sido observado en el grupo no-*americanum*, donde la presencia de grupos de morfoespecies no quedaron reflejados en su mayoría en los árboles filogenéticos a través de los clados producidos. No obstante, a partir de las relaciones filogenéticas basadas en el uso de la región D2D3 se detectó un clado bien delimitado y caracterizado por estar compuesto por especies que en su mayoría pertenecen al Grupo 5 (es decir, especies caracterizadas por la presencia de diferenciación uterina mediante el órgano "Pseudo-Z" junto con la presencia de espinas en la parte tubular del útero en el aparato reproductor femenino en las hembras adultas (Loof y Luc 1990). Estos resultados concuerdan con lo anteriormente descrito en estudios previos (Gutiérrez-Gutiérrez 2011, Zasada *et al.* 2014, Palomares-Rius *et al.* 2017a, 2017b). En resumen, aunque en el **Capítulo 3 ("Molecular phylogenetic analysis and comparative morphology resolve two new species of olive-tree soil related dagger nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) from Spain" y 4 ("Remarkable diversity and prevalence of dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches")** se puede consultar con un mayor detalle los aspectos relacionados en este sentido, un mayor esfuerzo científico debe de realizarse con el fin de encontrar procesos antropogénicos que pudieran estar detrás de los procesos de evolución en la familia Longidoridae.

Como se ha expuesto anteriormente, los marcadores ribosómicos han mostrado ser excelentes marcadores diagnósticos para especies de longidóridos en la mayoría de los casos estudiados. En un grupo de especies de difícil diagnóstico morfológico como es el grupo *americanum* del género *Xiphinema* caracterizado por presentar una notable plasticidad fenotípica entre especies, las relaciones filogenéticas basadas en la región

D2-D3 reveló dos clados bien soportados (**Capítulo 2, "Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematode: Longidoridae) as inferred from morphometrics and molecular markers").** Sin embargo, este marcador no diferenció a algunas de las especies, mientras que la región ITS fue más informativa para la confirmación molecular como ya ha sido descrito en previos estudios (Gutiérrez-Gutiérrez *et al.* 2012, Zasada *et al.* 2014). En consecuencia, la identificación polifásica basada en una estrategia integradora de combinación de técnicas moleculares con morfología y medidas ha demostrado ser una herramienta eficiente y confiable para la identificación de nematodos dentro de este grupo (Decraemer y Robbins 2007, Gutiérrez-Gutiérrez *et al.* 2010, 2012, Meza *et al.* 2011). No obstante, hasta el momento el empleo de dichos marcadores proporciona una herramienta adicional pero no una alternativa independiente al análisis exhaustivo de la morfología. En este sentido y al igual que en otros grupos de nematodos caracterizados por su elevada dificultad en su diagnóstico, la aplicación métodos estadísticos multivariantes han resultado una herramienta de elevada eficacia en el proceso de diagnóstico (Cantalapiedra-Navarrete *et al.* 2013, Tzortzakakis *et al.* 2016). Por lo tanto, la delimitación de especies pertenecientes al grupo *americanum* del género *Xiphinema* en el presente trabajo fue realizada en base a la aplicación integradora de métodos morfológicos, estadísticos y moleculares para desentrañar la diversidad de potenciales especies crípticas. Los resultados obtenidos en la delimitación del complejo de especies estrechamente relacionadas con la especie ampliamente distribuida como es *X. pachtaicum* certificó el uso del análisis multivariante como herramienta eficaz y complementaria en la identificación polifásica por medio de métodos morfológicos y moleculares en los NF (**Capítulo 2, "Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematode: Longidoridae) as inferred from morphometrics and molecular markers").**

En definitiva, la biodiversidad de especies de la familia Longidoridae detectada infestando suelos de olivo es realmente extraordinaria. A esto hay que añadir la descripción de un total de 14 nuevas especies distribuidas en 8 nuevos taxones para el género *Xiphinema* (*X. andalusiense*, *X. celtiense*, *X. iznajarense*, *X. mengibarense*, *X. macrodora*,

X. oleae, *X. plesiopachtaicum* y *X. vallense*) y 6 para *Longidorus* (*L. indalus*, *L. macrodorus*, *L. onubensis*, *L. silvestris*, *L. vallensis* y *L. wicuolea*). En particular, las nuevas especies descritas infestando suelos de olivo cultivado fueron un total de 5 y 4 taxones para *Longidorus* y *Xiphinema*, respectivamente. Por otro lado, las nuevas especies descritas asociadas al olivo silvestre fueron 4 y 6 taxones en el caso del género *Longidorus* y *Xiphinema*, respectivamente. Además de la descripción de nuevas especies, los datos de biodiversidad obtenidos en este trabajo han revelado nuevas citas de especies ya conocidas en asociación con el olivo cultivado así como silvestre. Por ejemplo, *L. vineacola*, una especie ampliamente distribuida por el continente europeo infestando suelos de ambientes naturales y agrícolas (Kruger 1988, Gutiérrez-Gutiérrez *et al.* 2013a), es por primera vez citada como especie capaz de parasitar las plantas de olivo tanto cultivado como silvestre. Un ejemplo en el caso del género *Xiphinema* es *X. cadavalense* (Bravo y Roca 1998), especie distribuida en la Península Ibérica y por primera vez asociada al olivo silvestre y cultivado. No obstante, son diversas las especies de esta familia que se citan por primera vez relacionadas con la planta huésped del olivo (silvestre y cultivado), cuya información al respecto puede ser encontrada en los **Capítulos 4 (Remarkable diversity and prevalence of dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches**) y **5** (**Unravelling the biodiversity and molecular phylogeny of needle neamatodes of the genus *Longidorus* (Nematoda: Longidoridae) in Olive and a description of six new species)** de la presente Tesis Doctoral.

Los resultados obtenidos en relación a la diversidad detectada entre el olivar silvestre y cultivado revelaron nuevos conocimientos sobre la influencia del ambiente natural y cultivado en la diversidad y distribución en las especies pertenecientes a la familia Longidoridae. Aunque se observaron diferencias en este sentido en el género *Longidorus*, el análisis de la biodiversidad en el género *Xiphinema* mediante la aplicación de índices de diversidad gamma (p. ej. índice de riqueza de especies, el índice Shannon o Hill entre otros) mostró una influencia significativa por parte del tipo de planta de olivo considerada (**Capítulo 4, "Remarkable diversity and prevalence of dagger nematodes of the genus**

***Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by integrative approaches"**). Aunque la diversidad de especies de nematodos longidóridos pertenecientes al género *Xiphinema* resultó ser extraordinaria, esta fue asociada principalmente al grupo no-*americanum* que mostró una amplia distribución en toda la superficie ocupada por la planta del olivo en Andalucía, pero principalmente asociada en las zonas donde el olivar silvestre mantiene su elevada presencia como es la provincia de Cádiz. Sin embargo, *Xiphinema pachtaicum*, perteneciente al grupo *americanum* y que resultó ser la especie más prevalente dentro del género *Xiphinema*, fue además una de las especies más abundantes y prevalentes de toda la nematofauna detectada en el olivo cultivado (ver **Bloque I, "Nematodos fitoparásitos asociados al cultivo del olivo en Andalucía"**). Otro resultado de interés subyace de la abundancia total detectada en cada punto de muestro, ya que ésta fue significativamente mayor para el grupo *americanum* siendo a su vez mayor para el olivar cultivado en comparación con el silvestre. Este aspecto podría estar relacionado con la elevada prevalencia y densidad detectada para la especie *X. pachtaicum*, lo cual podría apoyar la hipótesis que relaciona a esta especie con un problema real en el olivo cultivado (Peña-Santiago 1990). Por otro lado, los índices de diversidad utilizados en este estudio, especialmente en el caso del índice de riqueza de especies, revelaron un descenso de la diversidad en el olivar silvestre en contra del cultivado. Todo ello podría estar relacionado con el efecto del manejo agrícola sobre una amplia gama de propiedades ambientales relacionadas con el suelo y por consiguiente, en la ruptura del equilibrio en las comunidades de nematodos en comparación con los ecosistemas naturales (en este caso olivar silvestre) (Palomares-Rius *et al.* 2012, 2015, Sánchez-Moreno *et al.* 2015). En definitiva, el hecho de detectar el género *Xiphinema* con una extraordinaria diversidad, elevada prevalencia y abundancia en el olivo cultivado hace pensar que puedan estar detrás de una potencial reducción del crecimiento y producción en este cultivo en Andalucía. Todo puede entenderse por la existencia de estudios previos donde el género *Xiphinema* ha sido considerado como uno de los principales patógenos de suelo en el olivo en varios países incluyendo Chile o Estados Unidos, donde se estimó que la presencia de estas especies pueden ser responsables de una pérdida de producción entre el 5 y el 10% lo que

supondría una un coste cercano a los 39 millones de dólares (Hashim 1983, Koenning *et al.* 1999, Castillo *et al.* 2010, Ali *et al.* 2014).

Un resultado notable de la presente Tesis Doctoral y que está relacionado con la extraordinaria diversidad de especies de nematodos longidóridos en el olivar de Andalucía, es la identificación, y en este caso descripción taxonómica de dos especies del género *Xiphinema* caracterizadas por una singularidad extraordinaria en el campo de la fitonematolología, y en particular dentro de la familia Longidoridae (**Capítulo 3** "**Molecular phylogenetic analysis and comparative morphology resolve two new species of olive-tree soil related dagger nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) from Spain"**). La descripción de la nueva especie *Xiphinema oleae* ha resultado en un fenómeno no frecuente dentro del género *Xiphinema*, ya que la morfología que subyace de esta especie perteneciente al grupo no-*americanum* con la presencia de un "Órgano Z" verdadero determinando el aspecto de la diferenciación uterina (Kruger 1988), e incluyendo a este nematodo dentro del "Grupo de Morfoespecies 4", resulta en un hecho poco frecuente y por tanto de relevancia en este género dado el reducido número de especies con tales características (Loof y Luc 1990, Coomans *et al.* 2001). Por otro lado, la detección y posterior descripción de la nueva especie *Xiphinema macrodora* ha supuesto la reconsideración del tamaño nominal que caracteriza al género *Xiphinema* ya que esta especie presenta la mayor longitud del cuerpo y estilete (L = 8.7 mm; estilete = 206 μm) jamás descrita hasta la fecha, incrementando el rango de estas medidas en dicho género (Loof y Luc 1990, Loof *et al.* 1996, Lamberti *et al.* 2000, Coomans *et al.* 2001, Decraemer y Robbins 2007). Son varios los estudios previos que describen que la determinación de las dimensiones de los nematodos son procesos con origen determinista (Geraert 1968, Yeates 1986). De hecho, en el mismo punto de muestro se detectó la presencia de una especie de *Longidorus*, descrita como *Longidorus macrodorus*, que fue caracterizada como la especie del género *Longidorus* con la mayor longitud de estilete descrita en la bibliografía (ver **Capítulo 5 "Unravelling the biodiversity and molecular phylogeny of needle neamatodes of the genus *Longidorus* (Nematoda: Longidoridae) in Olive and a description of six new species").** Todo ello podría sugerir una adaptación ecológica por parte del nematodo condicionado por los factores

ambientales que describen el ecosistema en concreto donde fueron detectadas (Soetaert *et al.* 2002, Wardle 2002). En definitiva, todo lo comentado anteriormente fortalece la hipótesis comentada anteriormente y que describe el sur de la Península Ibérica como una posible área de especiación de la familia Longidoridae, dada no sólo por la extraordinaria diversidad en cuanto a número de especies sino a la variabilidad morfológica que muestra en dicha área.

Tal como se ha mostrado en la parte introductoria de esta Tesis Doctoral, el **Bloque III** ("**Descifrando la diversidad beta de nematodos fitoparásitos asociados al cultivo del olivo"**) fue diseñado para evaluar la relativa influencia de los factores ambientales y agronómicos en la distribución espacial de las comunidades de NF en el olivo cultivado en Andalucía. En este sentido, son diversos los estudios que han encontrado asociaciones entre factores ambientales y agrícolas con la distribución de estos organismos en el cultivo del olivo en importantes zonas olivareras incluyendo Andalucía (Palomares-Rius *et al.* 2015, Sánchez-Moreno *et al.* 2015, Aït Hamza *et al.* 2017, Ali *et al.* 2017). Teniendo en cuenta que la biodiversidad del suelo no se distribuye homogéneamente en el espacio y que está estructurada por la conjunción de procesos estocásticos y deterministas (Hubbell 2001, Vellend 2010), resultaría fácil entender que los factores ambientales (es decir, deterministas) que determinan dicha estructura espacial también puedan estar influenciados por la escala espacial considerada (Hubbell 2001, Ettema y Wardle 2002, Vellend 2010). Es por ello que la fiabilidad de los resultados obtenidos en los estudios comentados anteriormente está supeditada a la heterogeneidad espacial de los factores ambientales que estructuran la variabilidad espacial de la biodiversidad de los NF en el ecosistema de estudio mediante técnicas de modelización (Wiegand *et al.* 2017). En este sentido son diversos los estudios que han demostrado estructuras espaciales en organismos que viven sobre el suelo, siendo muy poco el esfuerzo destinado a aquellos que habitan el suelo donde se incluyen los NF (Soininen 2016). Por otro lado, la mayoría de los estudios que podemos encontrar en la bibliografía destinados a descubrir asociaciones entre factores ambientales y agronómicos con la biodiversidad de NF asociados al cultivo del olivo han sido desarrollados utilizando índices alfa o gamma como medidas de diversidad. Realizando una breve revisión bibliográfica en este sentido, son

diversos los inconvenientes que podemos encontrar con la aplicación de estos índices en comparación de aquellos que miden la diversidad beta, ya que ignoran la identidad de las especies (Tuomisto 2010) y por tanto, no permiten testar hipótesis sobre los procesos ecológicos a escala local y regional que impulsan los patrones de diversidad de especies a través del espacio y tiempo (Anderson *et al.* 2011). En definitiva, la aplicación de la diversidad beta como medida de la nematofauna detectada en este trabajo aumentará la fiabilidad sobre los factores ambientales que potencialmente pueden determinar la variabilidad de los ensamblajes de las comunidades de especies de NF en el cultivo del olivo en Andalucía.

En la **"Introducción y Objetivos"** de este trabajo se expusieron varias preguntas claves que no habían sido consideradas hasta la fecha en los estudios encontrados en la bibliografía que tienen como objetivo finalista encontrar asociaciones significativas entre gradientes ambientales y factores agrícolas con la distribución espacial de NF asociados al olivo cultivado. Si recordamos estas cuestiones eran las siguientes: ¿cuál es el papel de los patrones espaciales en la variación de las comunidades de NF?, o ¿está la influencia de los factores ambientales espacialmente estructurada? Además, si añadimos a estas cuestiones la cuantificación de los procesos estocásticos en la variabilidad espacial de las comunidades de NF incrementaríamos el conocimiento sobre los factores reales que afectan a tales patrones espaciales (Vellend 2010). La respuesta a estas cuestiones está relacionada con la incorporación de la fluctuación espacial mediante covariables a partir de la distribución espacial que subyace del diseño del muestreo (Dray *et al.* 2006). Existen varias metodologías han sido desarrolladas en los últimos años, aplicándose en este estudio aquella que está basada en el método de coordenadas principales de matrices de vecinos cercanos (PCNM) (Borcard y Legendre 2002). Este método crea variables espaciales a partir de la descomposición espectral de las relaciones espaciales entre los puntos de muestro el cual ha sido ampliamente utilizado en organismos animales y plantas (Borcard *et al.* 2004, Legendre *et al.* 2009), siendo su aplicación escasa para aquellas especies que habitan el suelo como son los NF.

Por otro lado, en este trabajo se ha determinado la disimilaridad espacial de las comunidades de NF identificadas entre los puntos de

muestro como la "varianza total de la comunidad" (diversidad beta; (Legendre y De Cáceres 2013), lo que permitió determinar la influencia ecológica de las unidades de muestro (índice LCBD) y de cada especie (índice SCBD) en función de su contribución en la variación total observada entre las comunidades de especies en cada punto de muestreo. Es decir, con objeto de identificar áreas y especies de particular interés, la diversidad beta se dividió en dos índices relacionados con la contribución de los puntos de muestreo (LCBD) y las especies (SCBD) en la variación de comunidades de NF. Por otro lado, es necesario indicar que en este estudio también se utilizó el índice de riqueza de especies como medida alternativa de la biodiversidad, lo cual nos permitió comparar nuestros resultados con los obtenidos en estudios previos, además de realzar la importancia de incorporar la diversidad beta como medida de la biodiversidad en los estudios de distribución de comunidades de parásitos en ecosistemas agrícolas. Una amplia descripción detallada sobre la metodología aplicada en este sentido puede ser consultada en el **Capítulo 6** (**Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olives in southern Spain**).

Además de la incorporación de las variables espaciales, el presente trabajo incluyó una amplia gama de factores potenciales para determinar la distribución espacial de las comunidades de NF identificadas en el **Capítulo 1** (**Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in southern Spain**). Por ello y con el objetivo finalista de determinar el efecto del ambiente y las prácticas agrícolas, se consideraron un total de 52 variables en este estudio (ver **Capítulo 1**), las cuales fueron distribuidas en tres bloques incluyendo clima (donde además de variables climáticas se tomaron aquellas que describen la orografía), suelo y manejo agronómico. En definitiva, en este estudio se evaluó la influencia de variables ambientales (suelo, clima y manejo agronómico) y de la estructura espacial sobre la variabilidad espacial de la riqueza de especies y la diversidad beta de NF infestando los suelos del olivar en Andalucía. Para ello, se han utilizado técnicas de partición de la varianza con el objetivo de evaluar las contribuciones únicas y compartidas de los diferentes bloques de variables, así como la autocorrelación espacial tanto endógena como exógena

(Borcard *et al.* 1992). Este método también permite evaluar la influencia relativa de los procesos estocásticos y deterministas que determinan la estructura espacial de las comunidades de especies, habiéndose utilizado tanto en animales y plantas (Svenning *et al.* 2004, Legendre *et al.* 2009), así como en organismos que viven bajo el suelo (Dumbrell *et al.* 2009).

Tal y como se ha expuesto anteriormente, la estabilidad de las comunidades de NF está determinada por la estabilidad del entorno abiótico, las interacciones entre los componentes bióticos, incluidos el huésped, así como la solidez y el equilibrio de la propia comunidad. Además de estos procesos deterministas, la variación en las comunidades de NF también puede estar impulsada por procesos neutros relacionados con las fluctuaciones aleatorias en la abundancia de especies, la limitación de la dispersión o la demografía aleatoria (Vellend 2010). Es decir, tanto los procesos determinísticos como los estocásticos conforman simultáneamente la estructura ecológica de las comunidades (Adler *et al.* 2007). En nuestro estudio, la partición de la varianza reveló un papel prominente de los procesos estocásticos en la estructuración de las comunidades de NF, lo que concuerda con la gran heterogeneidad detectada en la variación espacial de las comunidades de especies. Es decir, los patrones observados a lo largo del área de estudio mediante los mapas de riqueza de especies y diversidad beta mostraron una notable variabilidad entre las 376 parcelas comerciales muestreadas, lo que podría indicar que esta heterogeneidad no estuviera vinculada a procesos ecológicos de origen determinista. La baja influencia de los procesos deterministas podría estar asociada al efecto que producen los sistemas agrícolas en el suelo, frecuentemente perturbados (Ferrenberg *et al.* 2013). De hecho, estudios realizados en ambiente natural (es decir, no cultivado) describieron que la variación de la comunidad de nematodos fue dominada por procesos determinas en vez de estocásticos (Moroenyane *et al.* 2016). Otra razón podría estar relacionada con la configuración espacial y su influencia en los factores ambientales utilizados en el estudio (Smith y Lundholm 2010). En cualquier caso, cabe señalar que, aunque los procesos deterministas potencialmente no medidos fueran incorporados en los procesos de modelización (es decir, patrones de diversidad microbiana o depredadores y antagonistas de nematodos), es evidente que los procesos estocásticos seguirían dominando la variación de las

comunidades de NF. No obstante, se necesitarían más estudios basados en técnicas alternativas de partición de la varianza, así como la incorporación de variables que describan las interacciones bióticas para testar dicha hipótesis. Además, la partición de la diversidad beta filogenética y funcional puede proporcionar información adicional sobre el efecto relativo del componente determinista y estocástico, así como nuevos conocimientos sobre los procesos evolutivos que estructuran las comunidades de NF (Wang *et al.* 2013).

Los resultados obtenidos en el **Capítulo 6** (**Spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olives in southern Spain**) mostraron que la estructura espacial y las propiedades del suelo fueron los factores más importantes que determinan la variación de las comunidades de especies (diversidad beta) y en la variación de la riqueza de especies de la nematofauna identificada a través del área del cultivo del olivo analizada. Un resultado sorprendente dada su oposición con lo descrito en la bibliografía en este sentido, es la influencia escasa por parte del manejo agronómico en la riqueza de especies, siendo nula en la diversidad beta. Aunque se procederá a discutir brevemente las posibles razones que subyacen de este singular resultado, hay que recordar que en este estudio se han tenido en cuenta multitud de variables relacionadas con el manejo agronómico incluyendo tanto características de la propia planta (p.ej. edad, cultivar de olivo, etc.) como de las prácticas agrícolas efectuadas en la parcela (p.ej. densidad de plantación, régimen de riego, etc.) (ver **Capítulo 1** "**Data on spatial structure and soil properties shape local community structure of plant-parasitic nematodes in cultivated olive in southern Spain"**). Teniendo en cuenta esto, la baja y/o nula influencia de las variables agronómicas se considera un resultado de elevada relevancia en el campo de la Nematología, ya que desde la publicación del libro "Ecology of plant-parasitic nematodes" (Norton 1978) a fecha de hoy, tradicionalmente, la planta huésped y el manejo agrícola han sido considerados como los impulsores más importantes de las poblaciones de NF (Norton 1978, 1989, Freckman y Caswell 1985, Neher 2010, Palomares-Rius *et al.* 2012, 2015, Sánchez-Moreno *et al.* 2015, Ali *et al.* 2017). Por otro lado, la influencia del bloque de variables relacionado con el ambiente (es decir, clima y topografía) mostró una influencia residual en

los dos índices de biodiversidad usados en este estudio, observándose un fuerte gradiente espacial especialmente para este tipo de variables (aunque de forma generalizada) en la influencia sobre la variación de las comunidades de NF. Dada la escasa influencia de este componente, los aspectos más relevantes en este sentido no serán expuestos a continuación, aunque podrán consultarse de manera más detallada en el **Capítulo 6.**

Como ya se ha indicado nuestro estudio destaca la fuerte influencia de los factores del suelo en la variabilidad espacial de las comunidades de NF en el cultivo del olivo en Andalucía. Esto podría implicar la heterogeneidad del suelo puede interpretarse como la presencia de numerosos microhábitats que funcionan como pequeños ecosistemas para las comunidades de estos organismos (Nielsen *et al.* 2014). Además, nuestros resultados con respecto a la variación explicada de manera explícita por el componente del suelo mostraron una mayor dependencia de la diversidad beta que la riqueza de especies. Esto sugiere que las características del suelo podrían inducir principalmente cambios estacionales en la abundancia de los nematodos. Por lo tanto, podríamos deducir que la estructura del suelo condiciona los patrones de ensamblajes en las comunidades de NF independientemente de los gradientes ambientales y las interacciones con la planta huésped. En general, las variables de suelo que mostraron una mayor influencia fueron las propiedades químicas, incluyendo el contenido de CEC, Na y Mg, el pH del suelo y la disponibilidad de P; influencia que ya ha sido descrita en la bibliografía (Norton *et al.* 1971, Norton 1989, Mateille *et al.* 2014, Palomares-Rius *et al.* 2015). Cabe destacar la fuerte influencia de la disponibilidad de P en la variación de la riqueza de especies en contra de la diversidad beta. Aunque la disponibilidad de P se ha identificado como un factor determinante en la abundancia de los NF (Norton 1978), otros estudios han revelado efectos de filtrado en el tamaño de los nematodos del suelo por gradientes de P (Mulder 2010). Por tanto, la variabilidad en la concentración de este elemento podría estructurar comunidades de nematodos dependiendo del tamaño de estos, lo que podría explicar su influencia en la riqueza de especies dada la alta variabilidad en el tamaño que caracteriza la nematofauna detectada en este estudio (Gaugler y

Bilgrami 2004) (ver la variabilidad en la biomasa de cada especie en el **Capítulo 1**).

Nuestro estudio también mostró que la estructura espacial es otro de los componentes de elevada importancia en la variación de las comunidades de NF en el cultivo del olivo en Andalucía. Este hecho nos permite deducir que las variables espaciales por sí mismas son excelentes y potenciales descriptores de la distribución espacial de especies de nematodos dado que ésta presentó una fuerte estructura espacial (Dray *et al.* 2006). A diferencia de los sistemas sobre el suelo, pocos estudios se han centrado en determinar la contribución relativa de la estructura espacial en los patrones de diversidad en sistemas subterráneos (Jiménez *et al.* 2014, Xia *et al.* 2016). No obstante, estos estudios describieron también una fuerte influencia del componente espacial, explicando además una gran proporción de la variación de las comunidades de organismos de suelo como el observado en nuestro estudio. Es necesario recordar que la influencia pura del componente espacial puede representar variables que no se han tenido en cuenta en el estudio (De Cáceres *et al.* 2012). Sin embargo, la influencia relativa entre ambiente y estructura espacial detectada puede considerarse un efecto fiable dado la amplia gama de variables que se han tenido en cuenta en este estudio (Anderson *et al.* 2011). No obstante, el componente espacial puede representar la influencia de la limitación en la dispersión que caracteriza a los nematodos en el ecosistema del suelo (De Cáceres *et al.* 2012). En un contexto tridimensional, la capacidad de migración por parte del nematodo en el suelo no está clara, aunque está limitada por razones intrínsecas de la especie del nematodo así como de factores ambientales que caractericen el hábitat del suelo (Gaugler y Bilgrami 2004). En este sentido, varios estudios realizados en condiciones controladas testaron la capacidad de movimiento de varias especies de NF. Por ejemplo, en especies ectoparásitas migratorias como *Longidorus elongatus* y *Xiphinema diversicaudatum* la distancia máxima que pudieron desplazarse fue de 10 cm en un mes, aunque este periodo de tiempo estuvo influenciado en función de las condiciones ambientales en las que fue efectuado el experimento, llegando a superar los 10 cm en aproximadamente 5 horas (Gaugler y Bilgrami 2004). Por otro lado, las distancias de migración a gran escala en los nematodos están relacionadas a factores externos tales

como la actividad humana (p.ej. maquinaria agrícola, material vegetal de propagación, etc.), animal o a través de corrientes de agua y aire (Castillo *et al.* 2010, Neher 2010). No obstante, en este estudio se incluyó una variable que caracterizaba el origen del agua de riego incluyendo agua subterránea o superficial (aumentándose en este caso la probabilidad de migración), el cuál no se ajustó en los procesos de modelización y por tanto, no presentó efecto significativo en la variación de las comunidades de los NF.

Como ya se ha indicado, el papel del manejo agronómico en la diversidad de los NF detectados infestando los suelos del olivar en Andalucía fue en contraposición con lo descrito por la bibliografía. En los últimos años, ha aumentado el interés por evaluar la influencia de la activada humana (p.ej. prácticas agrícolas) en el ecosistema en relación con el impacto en los patrones de diversidad de los nematodos del suelo dada su excelente capacidad como bioindicadores de la calidad del suelo (Freckman y Caswell 1985, Yeates y Bongers 1999, Sánchez-Moreno *et al.* 2009, Neher 2010, Zhang *et al.* 2017). Concretamente en el agroecosistema del olivar ha sido ampliamente documentado que tanto la abundancia como la dinámica de las poblaciones de NF responden a la aplicación de diferentes tipos de manejo agrícola (Castillo *et al.* 2010, Palomares-Rius *et al.* 2015, Sánchez-Moreno *et al.* 2015, Ali *et al.* 2017). En contraposición con esta visión ampliamente aceptada por la comunidad científica, los resultados obtenidos en este estudio no indicaron un efecto puro por parte de las variables relacionadas con el manejo agronómico en la variación de la composición de las comunidades de especies (diversidad beta). Una posible explicación estaría relacionada con la no incorporación de la estructura espacial en los estudios previos en la bibliografía, por lo que el efecto de las prácticas agrícolas podría estar espacialmente estructurado (Vleminckx *et al.* 2017). Otra posible razón podría estar relacionada en que los estudios previos no se basaron en la diversidad beta sino en la aplicación de índices gamma y/o alfa como medidas de la biodiversidad de las comunidades de NF. De hecho, nuestros resultados revelaron cierto efecto puro por parte del componente del manejo agronómico en la variación de la riqueza de especies (ver **Capitulo 6**). La influencia pura del manejo agronómico en la riqueza de especies fue determinada fundamentalmente por la influencia de las variables que

caracterizaban la edad de la plantación del olivar, la presencia de riego y el manejo bajo la copa del árbol. El efecto de la edad de la plantación ya ha sido descrito en estudios previos (Zhang *et al.* 2015). No obstante, la influencia de la edad del árbol podría estar correlacionada con otras prácticas agrícolas tales como la densidad de la plantación (Rallo *et al.* 2013). Sin embargo, la variable que caracteriza la densidad de la plantación del olivar no quedo retenida en los procesos de modelización. Por tanto, la influencia de la edad podría estar asociada con las dimensiones de la copa del árbol y su efecto sobre las condiciones ambientales del suelo bajo esta y el impacto indirecto sobre la diversidad de los nematodos (Caldeira *et al.* 2014). Otro factor de influencia significativa sobre la riqueza de especies fue la presencia de riego en la plantación de olivar, ampliamente citado en la literatura (Neher 2010). Finalmente, y como era de esperar, la presencia de cubierta vegetal bajo la copa del árbol también mostró un efecto significativo en la riqueza de especies. Sin embargo, dicha influencia en la diversidad beta se mostró estructurada espacialmente, posiblemente correlacionada con otros factores edáficos y/o climáticos (ver **Capítulo 6**). La influencia pura de cubierta natural bajo la copa del árbol en la riqueza de especies confirma la selección relativa de especies de NF en base a la presencia de plantas herbáceas ajenas al cultivo del olivo (Castillo *et al.* 2010, Neher 2010, Palomares-Rius *et al.* 2015).

Aunque un mayor detalle puede encontrarse en el **Capítulo 6**, nuestro estudio reveló diferencias sustanciales entre los efectos de las variables consideradas en este estudio entre la diversidad beta y la riqueza de especies. En primer lugar, la variación explicada sobre la riqueza de especies fue superior que para la diversidad beta, lo que va en contra del patrón generalizado y descrito en los estudios realizados sobre comunidades de especies que viven sobre el suelo (Legendre *et al.* 2009, Punchi-Manage *et al.* 2014). Una posible explicación podría estar relacionada con la complejidad del ecosistema del suelo y su efecto estocástico en la abundancia de los nematodos (Ettema y Wardle 2002). En segundo lugar y ya mencionado, el no efecto puro (influencia no correlacionada con otros factores ambientales ni espacialmente estructurada) del manejo agronómico sobre la diversidad beta. Y en tercer lugar, la influencia pura del componente del suelo fue mayor en la

diversidad beta que en la variación de la riqueza de especies. Es evidente que la variabilidad en la abundancia de especies de NF está estrechamente determinada por los gradientes edáficos. No obstante, la variabilidad de dichas propiedades del suelo podrían estar atribuidas indirectamente a la aplicación de diferentes prácticas agrícolas (Sánchez-Moreno *et al.* 2015). Nuestros resultados apoyan esta hipótesis ya que el manejo agronómico no mostró una influencia pura sobre la diversidad beta (si recordamos fue calculada a partir de la biomasa (abundancia) de especies detectada en cada punto de muestro). Por el contrario, este componente si mostro un efecto puro sobre la riqueza de especies. Como ya se ha indicado, este resultado era lo esperado, dada la gran sensibilidad de ciertas especies de NF a la aplicación de diferentes prácticas agronómicas (Castillo *et al.* 2010, Neher 2010, Palomares-Rius *et al.* 2015). Este hecho podría deberse la existencia de diferentes estrategias reproductivas, así como una amplia variedad de formas de parasitismo dentro de los NF (Gaugler y Bilgrami 2004). Por lo tanto, las perturbaciones causadas por la aplicación de diferentes prácticas agrícolas podrían tener efectos diferenciales en ciertos grupos o especies de NF.

La variabilidad en los ensamblajes de las comunidades de nematodos es ampliamente reconocida como un potente indicador de las condiciones generales del suelo (Neher 2001, Sánchez-Moreno *et al.* 2009, Ferris 2010). El grado de variación de especies concretas en el área de estudio no tiene la misma interpretación que el concepto de “especies indicadoras” de características ecológicas en los ecosistemas (Legendre y De Cáceres 2013). Sin embargo, la contribución de cada especie en la variación total de la comunidad de especies en la zona de estudio (SCBD) se correlacionó significativamente con la prevalencia y el rango de densidad de los NF, sugiriendo que el índice SCBD podría estar relacionado con la posición del nicho ecológico como ocurre en otros sistemas (Heino 2005, Heino y Grönroos 2017). Por lo tanto, las especies de NF que exhiben una mayor prevalencia y muestran una notable variación en la abundancia en la que son detectadas entre los puntos de muestro podrían considerarse como indicadores adecuados de fluctuaciones ambientales específicas en los agroecosistemas (Heino y Grönroos 2017). De acuerdo con los resultados obtenidos para el índice SCBD (ver **Capítulo 1**), este estudio encontró que la mayoría de especies de nematodos que exhibieron los

valores más elevados para éste índice pertenecían a los géneros *Helicotylenchus* y *Xiphinema*. El hecho de las especies de ambos géneros sean caracterizados como especies con estrategia-*K* de supervivencia unido a que la mayoría de sus especies sean ectoparásitos migratorios, hace que ambos géneros puedan considerarse "grupos funcionales" en el sentido de predecir fluctuaciones en las propiedades ecológicas del suelo y por tanto, potenciales bioindicadores en ecosistemas agrícolas como ya ha sido descrito por varios autores (Bongers 1990, Yeates y Bongers 1999).

Esperábamos que la singularidad ecológica de las unidades de muestreo en términos de la composición de la comunidad (LCBD) estuviera influenciada por la riqueza de especies y la abundancia de nematodos. Sin embargo, y en contraposición a lo observado en estudios efectuados en sistemas sobre el suelo (Heino y Grönroos 2017), estas relaciones no fueron observadas en nuestro estudio. A pesar de ello, encontramos que el índice LCBD se relacionó positivamente con la biomasa total de nematodos detectada en cada punto de muestro (ver **Capítulo 6**). Dado que la biomasa entre especies de nematodos puede variar considerablemente en función de su tamaño (Gaugler y Bilgrami 2004), la biomasa total de NF puede ser muy variable entre dos sitios dependiendo de la identidad de las especies que compongan la comunidad en el supuesto de que la abundancia de especies sea similar. Por lo tanto, la singularidad ecológica de cada sitio (LCBD) puede dar lugar a cambios claramente perceptibles en las comunidades de NF en función del tamaño de la especie, en función de la estrecha relación entre dicho aspecto y los gradientes ecológicos (Mulder 2010).

Por otro lado, la mayoría de las parcelas muestreadas con valores altos para el índice LCBD se agruparon espacialmente en dos áreas claramente separadas (ver **Capítulo 6**). Esto podría sugerir que los procesos subyacentes que determinan la singularidad de los puntos de muestro podrían estar relacionados tanto por gradientes ecológicos como componente espacial. De hecho, nuestros resultados mostraron que la influencia de las variables ambientales (fundamentalmente relacionadas con el suelo) fue mayor que aquella descrita para el componente espacial puro. No obstante, un grupo de variables relacionadas con el manejo agronómico también mostró una influencia significativa en la variación

espacial del índice LCBD, al igual que ocurrió con la riqueza de especies. Este resultado respalda la hipótesis de que la aplicación de diferentes prácticas agronómicas podría tener un efecto de selección para ciertas especies de NF en el cultivo del olivo (Castillo *et al.* 2010, Neher 2010, Palomares-Rius *et al.* 2015). En general, sugerimos que la naturaleza excepcional de la composición de especies de los sitios de muestreo está más influenciada por las variables de hábitat que por los rasgos biológicos intrínsecos de las especies. Por lo tanto, los estudios futuros basados en la comparación de estas características biológicas de los nematodos y la singularidad en los ensamblajes en la composición de las comunidades de especies en los puntos de muestro nos permitirá una mejor comprensión sobre la agregación de especies de NF en la red trófica del suelo. En un contexto fitopatológico, otros estudios también podrían basarse en la variación espacio-temporal de los valores de LCBD mediante la aplicación de diferentes prácticas agronómicas con el objetivo de determinar qué estrategias de manejo podrían ser más efectivas en el control de NF en el cultivo del olivo (Legendre y Gauthier 2014).

En definitiva, el desafío que desde el punto de vista de unión de ámbitos científicos alejados y a la vez estrechamente relacionados como son la taxonomía, fitopatología y la ecología, ha constituido que la resolución de la investigación planteada, la amplitud de la misma y el abordaje con enfoque multidisciplinar, acentúan el interés de esta Tesis Doctoral en cuanto a los conocimientos científicos proporcionados en el ámbito de la Fitonematología en general. Hasta la fecha la falta de información en relación sobre la diversidad de NF asociada al cultivo del olivo en una importante zona olivarera como es Andalucía, podría estar detrás de la falta de consideración de estos organismos como un problema sanitario de relevancia en el cultivo del olivo en esta zona. Sin embargo, los resultados obtenidos en esta investigación en cuanto a la extraordinaria diversidad de estos organismos, así como su amplia distribución y abundancia podría revertir esta situación y por tanto, considerar a estos organismos como un potencial problema fitosanitario que puede ser reforzado por la importante transformación que está sufriendo el sector del olivar en Andalucía. En este sentido, la presente Tesis Doctoral proporciona nuevos conocimientos para mejorar las prácticas de manejo y anticipar respuestas a la posibilidad potencial de la presencia de

enfermedades emergentes ante los cambios tecnológicos que está imponiendo la nueva olivicultura, así como a los cambios climáticos que pueden afectar a la severidad de estos organismos. Del mismo modo que el conocimiento de la biodiversidad es esencial para mantener el bienestar humano, desentrañar la diversidad de organismos potencialmente causantes de problemas fitosanitarios en un contexto agrícola es una de las claves del éxito que puedan tener las posibles medidas de control a efectuar (van der Putten *et al.* 2006). En este sentido, el carácter innovador de la presente Tesis Doctoral subyace en varios aspectos esenciales tales como: el enfoque sistemático en el diseño del muestreo abordado, siendo el estudio con el mayor esfuerzo en este sentido descrito hasta la fecha, lo que ha permitido obtener datos fiables sobre la biodiversidad de NF asociada a este cultivo en Andalucía; y la evaluación de la influencia relativa de los factores ambientales y agronómicos que determinan la distribución de dicha diversidad mediante métodos científicos de ámbitos ecológicos con escasa aplicación en los organismos de suelo y en particular, en los NF.

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CONCLUSIONES (CONCLUSIONS)

Unravelling of soil nematode biodiversity is an essential task in order to increase the knowledge about ecological phenomenon from the evolutionary, biogeographical and physical processes in soil ecosystems. In this regard, we aimed to unravel the diversity of plant-parasitic nematodes (PPN) associated with cultivated olive in southern Spain through the largest sampling effort on olive. We conducted a systematic survey comprising 376 commercial olive orchards covering the diversity of cropping systems that characterize the entire olive growing area of Andalusia, including agroforestry stands, traditional groves and new intensive orchards, as well as a wide range of ecological gradients related with topography, soil and climate. From this research, we got the following conclusions:

1. A total of 128 PPN species, belonging to 38 genera and 13 families, were recorded by using integrative taxonomy based on morphological and molecular approaches resulting in the greatest taxonomical diversity detected in cultivated olive. This research increases the number of PPN associated with olive, being estimated about 250 species worldwide. The three most prevalent families in cultivated olive from Andalusia were Tylenchidae, Paratylenchidae and Criconematidae, and the nematode families with the highest average nematode densities were Meloidogynidae, Hoplolaimidae and Paratylenchidae. And the family with the highest number of species was Longidoridae with a total of 28 species identified.

2. The PPN abundance in olive ranged from 7 to 19,796 nematode specimens per 500 cm^3 of soil. *Helicotylenchus oleae* and *Ogma rhombosquamatum* showed the highest nematode abundance with 19,796?? and 9,800 nematodes per 500 cm^3 of soil, respectively. The nematode species most prevalent were *Merlinius brevidens* (72.6%) and *Xiphinema pachtaicum* (70.4%).

3. We demonstrate the importance of using integrative taxonomic identification emphasizing the time-consuming aspect and difficulty of correct identification at species level within the family Longidoridae. In

addition, we provide a new insight in the identification of *Xiphinema americanum*-group species including statistical multivariate methods to the custom integrative taxonomical approach.

4. This research make available the first detailed analysis of the diversity and distribution of PPN belonging to Longidoridae infesting wild and cultivated olive in a wide-region in southern Spain as Andalusia, providing new insights of this family associated with olive in Mediterranean conditions. We reveal a remarkable diversity and distribution of longidorid species infesting soils of olives (cultivated and wild) in Andalusia with a total identification of 32 and 13 species for *Xiphinema* and *Longidorus*, respectively; and diversity indexes were significantly affected by olive type. This research provides a complete characterization of 15 new species belonging to the family Longidoridae, providing also molecular markers of known longidorid species for precise and unequivocal diagnosis in order to differentiate virus vector or quarantine species.

5. Local stochastic assembly processes dominate community structure of PPN infesting cultivated olives since more than two thirds of the variation in community composition and species richness remained unexplained. Additionally, their beta diversity was less structured by space and environmental factors as compared to other organisms studied before.

6. Spatial and soil variables were most important, whereas agronomic management practices showed less influence than expected for species richness, and no effect for beta diversity was detected. We found relatively high levels of shared contributions of the different sets of variables, especially with space, indicating spatial gradients in the environmental variables.

7. Species contributions to beta diversity (SCBD) were positively correlated with nematode prevalence and density range, suggesting that SCBD could be related with niche position as reported in other ecosystems.

8. In summary, novel insights revealed that the diversity of PPN was structured by the effect of environment and agronomic practices. In

addition, it also shows that beta diversity was less structured by space and environmental factors as compared to other organism types such as plants or other animal communities, which would allow to increase the reliability of the management practices of these parasites in agricultural ecosystems.